

METHODS AND COMPOSITIONS TO MODULATE EXPRESSION IN PLANTS

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Related Applications

This is a continuation-in-part of U.S. Application Serial No. 09/620,897, now pending, which claims priority, under 35 U.S.C. § 119(e), of U.S. Provisional Patent Application Serial No. 60/177,468, filed January 21, 2000. The disclosure of the above referenced applications is incorporated by reference in its entirety.

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Technical Field

The invention relates to the field of plant and agricultural technology. More specifically, the invention relates to the use of zinc finger proteins and fusions of said proteins to regulate gene expression and metabolic pathways in plants.

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Background Art

Genetic expression in general, and in plants in particular, is controlled at both transcription and translation levels. Regulation of transcription often involves regulatory factors which contain zinc finger domains of a particular family - e.g., domains which comprise approximately 30 amino acids containing two cysteine and two histidine residues folded around a zinc ion and providing an alpha helical recognition sequence specific at the "fingertip" for a particular 3-nucleotide sequence. The nature of such zinc fingers is set forth, for example, in WO 98/54311, the contents of which are incorporated herein by reference. As there are 64 possible three nucleotide targets for binding by zinc fingers, it is theoretically possible to design 64 individual zinc fingers, each of which would bind specifically to only one of the 64 possible triplets. By combining multiple zinc finger motifs, a larger target sequence could be bound specifically.

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It has been calculated that a nucleotide sequence containing only 18 nucleotides would serve as an unique address within even a 68 billion pair genome; thus, an 18

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nucleotide sequence would clearly serve as an unique target within the human genome (3.5 billion bp) or maize (2 billion bp). Thus, a hexadactyl zinc finger protein, properly designed, could target any arbitrary unique sequence within the human or maize genome. Considering the complexity of human and maize genome, this specially designed zinc finger protein could target any unique sequence within these and other organisms.

In the context of regulatory transcription factors, zinc finger domains which are responsible for specifically targeting a particular nucleotide sequence within a gene are generally coupled to additional amino acid sequences which serve to modulate expression either by activating (amplifying) or repressing it. Thus, typical transcription regulatory factors comprise both a zinc finger domain responsible for targeting the appropriate position of the genome and a functional portion which controls transcription of the gene once the fusion protein is bound.

Synthetic zinc finger proteins have been synthesized and found to have binding affinity similar to those found in native transcription factors. Further, zinc finger proteins have been designed which are specific for TGA or for one of the triplets of the formula GNN. Thus, zinc finger proteins can be designed to target unique sequences of the formula $(GNN)_6$ or sequences containing 18 nucleotides wherein some of the GNN triplets have been substituted by TGA. As the design of zinc finger proteins progresses, appropriate zinc finger domains can be designed for any desired target sequence.

There is evidence to show that specific synthetic zinc finger proteins can transiently regulate reporter gene, *e.g.*, luciferase, expression in cultured mammalian cells when fused with a transcriptional activation or repression domain (Liu et al., *Proc. Natl. Acad. Sci. USA*, 94:5525-5530 (1997); Wu et al., *Proc. Natl. Acad. Sci. USA*, 92:344-348 (1995); and Beerli et al., *Proc. Natl. Acad. Sci. USA*, 95:14628-14633 (1998)). However, there has been no data showing: (1) if these synthetic zinc finger proteins can be used to manipulate endogenous gene expression; and (2) if such transient regulation can be stabilized. There has also been no scientific evidence predicting if this technology will perform well in a whole living organism as it may in tissue culture system.

In addition to all these unknown factors, a plant cell is considered different than a mammalian cell in numerous aspects even though they share most of the basic features of living organisms. First, plant cells have different subcellular biological structures, such

as cell walls, which make the mechanism and procedure of transformation of foreign gene into plant cells significantly different from mammalian cells. Second, the genetic recombination mechanism and frequency in plant cells differ from that in mammalian cells as well. For any given transgene to be expressed and functional in any living cell, the very critical step is integration into host genome, the mechanism of which differs between plant and mammalian cells. Generally, plant cells have much lower integration frequency. Third, most plant cells have specialized metabolic pathway and enzymes catalyzing these pathways so that a gene functioning in a mammalian cell is not necessarily functional in a plant cell. Fourth, the preference of genetic codon usage is different amongst plant, mammalian, and other biological systems.

It is highly desirable to control expression of target genes in plants whether these genes are native to the plant or constitute modifications of the native plant genetic complement. The present invention provides such means, and is exemplified by the control of expression of three genes in plants: (1) the reporter gene luciferase in tobacco and maize cells; (2) the APETALA3 (AP3) gene in Arabidopsis plant, and (3) the gene encoding *myo* inositol 1-phosphate synthase (MIPS) in maize, all of which are representatives of economically valuable genes.

Thus, there remains a need for methods and compositions to control, at will, gene expression and other functions and activities in plants, plant tissues, and plant cells. The present invention address these and other related needs in the art.

Summary of the Invention

The invention relates to the field of plant and agricultural technology. In one aspect, the present invention is directed to a method to modulate the expression of a target gene in plant cells, which method comprises providing plant cells with a zinc finger protein, said zinc finger protein being capable of binding, and preferably, specifically binding, to a target nucleotide sequence, or a complementary strand thereof, within a target gene, and allowing said zinc finger protein binding to said target nucleotide sequence, whereby the expression of said target gene in said plant cells is modulated.

The zinc finger protein can be provided to the plant cells via any suitable methods known in the art. For example, the zinc finger protein can be exogenously added to the plant cells and the plant cells are maintained under conditions such that the zinc finger protein binds to the target nucleotide sequence and regulates the expression of the target gene in the plant cells. Alternatively, a nucleotide sequence, e.g., DNA or RNA, encoding the zinc finger protein can be expressed in the plant cells and the plant cells are maintained under conditions such that the expressed zinc finger protein binds to the target nucleotide sequence and regulates the expression of the target gene in the plant cells.

A preferred method to modulate the expression of a target gene in plant cells comprises the following steps: a) providing plant cells with an expression system for a zinc finger protein, said zinc finger protein being capable of binding, and preferably specifically binding, to a target nucleotide sequence, or a complementary strand thereof, within a target gene; and b) culturing said plant cells under conditions wherein said zinc finger protein is produced and binds to said target nucleotide sequence, whereby expression of said target gene in said plant cells is modulated.

Any target nucleotide sequence can be modulated by the present method. For example, the target nucleotide sequence can be endogenous or exogenous to the target gene. In a specific embodiment, the target nucleotide sequence is endogenous to the plant but is in a non-naturally-occurring location. The target nucleotide sequence can be located in any suitable place in relation to the target gene. For example, the target nucleotide sequence can be upstream or downstream of the coding region of the target gene. Alternatively, the target nucleotide sequence is within the coding region of the target gene. Preferably, the target nucleotide sequence is a promoter of a regulatory protein. Also preferably, the target nucleotide sequence comprises 3, 6, 9, 12, 15 or 18 nucleotides. More preferably, the target nucleotide sequence comprises 18 nucleotides and wherein the zinc finger protein is a hexadactyl zinc finger protein. Further preferably, the targeted nucleotide sequence is of the formula $(GNN)_n$, and wherein N is any one of the A, T, C or G and n is an integer from 1 to 6. More preferably, the targeted nucleotide sequence is of the formula $(GNN)_6$, and wherein N is any one of the A, T, C or G.

Plant cells containing any copy number of the target nucleotide sequence can be used in the present methods. For example, the plant cells comprising at least two copies of the same or different target nucleotide sequence can be used. Alternatively, each target nucleotide sequence can be located within a different target gene so that more than one different target genes can be modulated.

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Any target gene can be modulated by the present method. For example, the target gene can encode a product that affects biosynthesis, modification, cellular trafficking, metabolism and degradation of a peptide, a protein, an oligonucleotide, a nucleic acid, a vitamin, an oligosaccharide, a carbohydrate, a lipid, or a small molecule. Exemplary proteins or peptides include enzymes, transport proteins such as ion channels and pumps, nutrient or storage proteins, contractile or motile proteins such as actins and myosins, structural proteins, defense proteins or regulatory proteins such as antibodies, hormones and growth factors. Exemplary nucleic acids include DNA, such as A-, B- or Z-form DNA, and RNA such as mRNA, tRNA and rRNA. The nucleic acids can be single-, double- and triple-stranded nucleic acids. Exemplary vitamins include water-soluble vitamins such as thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, folate, vitamin B₁₂ and ascorbic acid, and fat-soluble vitamins such as vitamin A, vitamin D, vitamin E, and vitamin K. Exemplary lipids include triacylglycerols such as tristearin, tripalmitin and triolein, waxes, phosphoglycerides such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol and cardiolipin, sphingolipids such as sphingomyelin, cerebrosides and gangliosides, sterols such as cholesterol and stigmasterol and sterol fatty acid esters. The fatty acids can be saturated fatty acids such as lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid and lignoceric acid, or can be unsaturated fatty acids such as palmitoleic acid, oleic acid, linoleic acid, linolenic acid and arachidonic acid. In a specific embodiment, the target gene encodes a protein or an RNA. In another specific embodiment, the target gene is a reporter gene, e.g., luciferase, the AP3 gene or the *myo inositol 1-phosphate synthase* gene. In still another specific embodiment, the present method is used for treating a disorder in the plant cells, wherein the disorder is associated with abnormal expression of the target gene.

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When the target gene encodes a target protein, the present method can be used to modulate the expression of said encoded target protein. Expression of any target protein can be modulated by the present method in plant cells. The protein whose expression being modulated can be endogenous or exogenous to the plant cell. The modulation can be activation or inhibition.

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In a specific embodiment, the protein whose expression being modulated is an antibody. In another specific embodiment, the protein whose expression being modulated participates in a metabolic pathway or controls a metabolic pathway, *e.g.*, an anabolic or a catabolic pathway. The present method can be used for modulating metabolic pathways of any desirable molecules such as vitamins, taste molecules, *e.g.*, bad taste molecules, anti-oxidants, sugars and flavanoids. The metabolic pathway being modulated can be endogenous or exogenous to the plant cell. In still another specific embodiment, target gene encodes a structural protein, *e.g.*, an enzyme or a co-factor in a metabolic pathway, or a regulatory protein. In yet another specific embodiment, the metabolic pathway being modulated enhances an input or output trait in a plant or seed.

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Although in some cases, a zinc finger protein itself is sufficient for modulating gene expression, the zinc finger protein is preferably fused to a protein which activates or represses gene expression, *e.g.*, an activator domain of a regulatory protein or an active domain of a nucleic acid modifying protein.

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For modulating the expression of the target gene, the zinc finger protein can bind to the target nucleotide sequence within the target gene. Alternatively, the zinc finger protein can bind to the complementary strand of the target nucleotide sequence. Additionally, the zinc finger protein can specifically bind to an effector domain of the target sequence and whereby the expression of the target gene is modulated by competitive inhibition of said effector domain. In such case, the zinc finger protein preferably does not comprise an effector domain.

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The zinc finger protein used in the present methods can comprise a plurality of finger regions. The zinc finger protein can comprise linker regions among the plurality of finger regions. For example, the zinc finger protein used in the present method can contain any number of the 3-finger region. Preferably, the zinc finger protein can comprise at least two 3-finger regions that are separated and linked together with a linker

region. The linker region can be any suitable length, e.g., from about 2 to about 10 amino acid residues in length. Preferably, the linker region between any said two 3-finger region is about 5 amino acid residues in length.

In addition to the typical zinc finger domains, the zinc finger protein can further comprise other desirable domains such as effector domains active in the host plant cells. Any types of zinc finger protein can be used in the present method. But preferably, the zinc finger protein comprising a framework from a plant zinc finger protein can be used. Alternatively, synthetic zinc finger proteins or non-naturally-occurring zinc finger proteins can be used.

In a specific embodiment, the zinc finger protein used in the present methods is ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3, alone or fused to an effector domain.

In another specific embodiment, the zinc finger protein used in the present methods is not any zinc finger protein that is disclosed in U.S. Patent No. 6,140,466 or WO 98/54311, e.g., a zinc finger-nucleotide binding polypeptide variant comprising at least three zinc finger modules that bind to a target cellular nucleotide sequence and modulate the transcriptional function of the cellular nucleotide sequence, wherein the amino acid sequence of each zinc finger module that binds a target cellular nucleotide comprises two cysteines and two histidines whereby both cysteines are amino proximal to both histidines and wherein each of three modules of said variant has at least one amino acid sequence modification.

The present methods can be used to modulate gene expression in any plant cells, e.g., monocot or dicot plant cells. The plant cells can be in any suitable forms. For example, the plant cells can be included within an intact plant, and preferably the plant cells constitute all the cells of an intact plant. Alternatively, the plant cells can be contained in an *in vitro* culture, and preferably be cultured in planta. The plant cells can also be in the form of protoplasts or spheroplasts.

The present method can be used to achieve any desirable degree of the modulation of a target gene expression. Preferably, the modulation of the gene expression is at least two fold, e.g., at least five fold repression or at least two fold activation. Also preferably, the modulation changes the phenotype of the plant cells, the tissue(s) of the plant or the whole plant.

When the zinc finger protein is provided to plant cells with an expression system for a zinc finger protein, any suitable promoters can be used in directing expression of the zinc finger protein. The expression system can comprise a constitutive promoter or an inducible promoter. In addition, the zinc finger protein can be expressed transiently or 5 stably.

Preferably, the expression system can comprise a tissue-specific promoter. Also preferably, the expression of the zinc finger protein is controlled by a tissue-specific promoter and whereby tissue-specific modulation of the target gene expression is obtained. In this way, the zinc finger protein can be expressed in any desirable plant 10 tissue, such as calli, meristem, leave, root or organ explant in tissue culture.

Also preferably, the zinc finger protein can be expressed in a specific organelle, such as a mitochondria, a nucleus, a plastid or a vacuole. Exemplary plastids include chloroplast, leucoplast, aravloplast and chromoplast.

When the zinc finger protein is provided to plant cells with an expression system for a zinc finger protein, the nucleotide sequence encoding the zinc finger protein can be targeted to or stably integrated in a specific organelle. Such nucleotide sequence can be targeted to a specific organelle by any methods known in the art. For example, the zinc finger protein can be targeted to plastid via a plastid transit peptide, to chloroplast via a chloroplast transit peptide, to mitochondrial via a mitochondrial target peptide or to 15 nucleus via a nuclear targeting peptide.

When the zinc finger protein is provided to plant cells with an expression system for a zinc finger protein, the nucleotide sequence encoding the zinc finger protein can comprise preferred codons of the host plant, *e.g.*, preferred translational start codon of the host plant.

In another aspect, the present invention is directed to a method of modulating a 25 level of a compound in a plant cell, which method comprises expressing in a plant cell a zinc finger protein that binds or specifically binds to a target nucleotide sequence within a target gene to modulate expression of said target gene which is involved in a compound's metabolism in said plant cell, whereby the level of said compound in said plant cell is modulated. The level of any compound, *e.g.*, phytic acid, or target gene, *e.g.*, AP3, can 30 be modulated by the present method in plant cells. Preferably, the zinc finger protein

used in the present methods is not any zinc finger protein that is disclosed in U.S. Patent No. 6,140,466 or WO 98/54311, *e.g.*, a zinc finger-nucleotide binding polypeptide variant comprising at least three zinc finger modules that bind to a target cellular nucleotide sequence and modulate the transcriptional function of the cellular nucleotide sequence, wherein the amino acid sequence of each zinc finger module that binds a target cellular nucleotide comprises two cysteines and two histidines whereby both cysteines are amino proximal to both histidines and wherein each of three modules of said variant has at least one amino acid sequence modification.

In still another aspect, the present invention is directed to an expression vector comprising a nucleotide sequence encoding a zinc finger protein, said zinc finger protein is capable of binding or specifically binding to a target nucleotide sequence, or a complementary strand thereof, within a target gene whose expression is to be modulated by said zinc finger protein. A plant that is regenerated from a plant transformed with the above expression vector is also provided. Preferably, the zinc finger protein used in the present expression vectors is not any zinc finger protein that is disclosed in U.S. Patent No. 6,140,466 or WO 98/54311, *e.g.*, a zinc finger-nucleotide binding polypeptide variant comprising at least three zinc finger modules that bind to a target cellular nucleotide sequence and modulate the transcriptional function of the cellular nucleotide sequence, wherein the amino acid sequence of each zinc finger module that binds a target cellular nucleotide comprises two cysteines and two histidines whereby both cysteines are amino proximal to both histidines and wherein each of three modules of said variant has at least one amino acid sequence modification.

In yet another aspect, the present invention is directed to a genetically modified plant cell, which cell comprises an expression system for a zinc finger protein, said zinc finger protein is capable of binding, preferably specifically binding, to a target nucleotide sequence, or a complementary strand thereof, within a target gene whose expression is to be modulated by said zinc finger protein.

In yet another aspect, the present invention is directed to a zinc finger protein that is ZFPm1, ZFPm2, ZFPm3, ZFPm4, or ZFPAp3, preferably in combination with positive and negative regulating domains, or a fusion protein comprises a zinc finger of 2C7 and an effector domain of SID (2C7-SID fusion protein).

In yet another aspect, the present invention is directed to an isolated nucleic acid fragment comprising a sequence of nucleotides encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4, ZFPAp3 or a 2C7-SID fusion protein. Plasmids and cells containing the nucleic acid fragments and methods for producing ZFPm1, ZFPm2, ZFPm3, ZFPm4, ZFPAp3 proteins or 2C7-SID fusion proteins using the plasmids and cells are also provided.

5 In yet another aspect, the present invention is directed to an antibody that specifically binds to the above-described zinc finger protein.

10 In yet another aspect, the present invention is directed to a zinc finger protein comprising a zinc finger nucleic acid binding domain and an effector domain, wherein said effector domain comprises an active domain of a restriction enzyme, an active domain of a nucleic acid modifying protein, *e.g.*, a nucleic acid methylase, a label or a modification.

15 In yet another aspect, the present invention is directed to an assay method for determining a suitable position in a gene for regulation of expression in plant cells, which method comprises: a) providing a target gene which contains a nucleotide sequence encoding a reporter protein within the coding region of said target gene and a target nucleotide sequence at a predetermined location within said target gene; b) contacting said target gene with a regulatory factor comprising a zinc finger protein specific for said target nucleotide sequence; and c) assessing the level of expression of said reporter gene in the presence and absence of said contacting; wherein a change in the level of expression of said reporter gene in the presence as opposed to the absence of said contacting identifies said position of said target nucleotide sequence as a position suitable for controlling expression of said target gene in plant cells.

20 Preferably, the zinc finger protein used in the present assay methods is not any zinc finger protein that is disclosed in U.S. Patent No. 6,140,466 or WO 98/54311, *e.g.*, a zinc finger-nucleotide binding polypeptide variant comprising at least three zinc finger modules that bind to a target cellular nucleotide sequence and modulate the transcriptional function of the cellular nucleotide sequence, wherein the amino acid sequence of each zinc finger module that binds a target cellular nucleotide comprises two cysteines and two histidines whereby both cysteines are amino proximal to both

histidines and wherein each of three modules of said variant has at least one amino acid sequence modification.

Brief Description of the Drawings

- 5 Fig. 1 A Constructs for analyzing activation of reporter gene in maize HE89 (F19556) cells.
- 10 Fig. 1 B Activation of reporter gene in maize HE89 (F19556) cells. Maize Ubiquitin promoter was used to drive the expression of 2C7-VP64 fusion protein (activator). Reporter I (p5'C7F) is generally activated 150 to 250 fold. Reporter II activation is between 40 to 70 fold.
- 15 Fig. 2 Reporter plasmids for evaluating different binding site positions.
pAluc: Full length CsVMV; p5'C7A: Full length CsVMV with 6x2c7 binding sites at the 5' end; p3'C7A: Full length CsVMV with 6x2c7 binding sites at the 3' end of the 5'UTR; pC78E: CsVMV with 6x2c7 binding sites replacing -112 to -63; p5'C7C: CsVMV (-222 to +72) with 6x2c7 binding sites at the 5' end; p5'C7D: CsVMV (-178 to +72) with 6x2c7 binding sites at the 5' end; p5'C7F: CsVMV (-112 to +72) with 6x2c7 binding sites at the 5' end; pc7rbTATA: Minimal promoter with a TATA box with 6x2c7 binding sites at the 5' end; prbTATA: Minimal promoter with a TATA box with no ZFP binding sites.
- 20 Fig. 3 Zinc finger protein-effector fusion constructs for evaluation of the position effect of ZFP binding site in tobacco cells. 2C7-SID: 6 finger ZFP fused to the Sin3 interaction domain (SID); 2C7-SKD: 6 finger ZFP fused to the super krab domain (SKD); 2C7-VP64: 6 finger ZFP fused to 4 repeats of the minimal VP16 activation domain (VP64); C7-VP64: 3 finger ZFP fused to 4 repeats of the minimal VP16 activation domain (VP64); C7-GFP: 3 finger ZFP fused to the Green Fluorescent Protein (GFP); GFP: Free GFP.
- 25 Fig. 4 A Effects of zinc finger protein binding site on reporter gene expression levels and activation and activation of reporter gene with 3-(blue) or 6-zinc (cross hatch) finger proteins. A) Effects of different binding site
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positions on activation by a 3-finger effector (c7-vp64, solid bars) or a 6-finger protein (2c7-vp64, striped bars).

- 5 Fig. 4 B Activation of a minimal TATA containing reporter.
- 10 Fig. 5 Repression of reporters with 3- or 6-finger zinc finger fusion proteins.
- 15 Fig. 6 DNA recognition helix sequences of ZFPm1, ZFPm2, ZFPm3, ZFPm4, and ZFPAp3.
- 20 Fig. 7 ELISA analysis of ZFPm1 DNA-binding specificity. Six-finger protein ZFPm1 was purified through affinity column from as MBP fusion protein from *E. coli*. Specificity of binding was analyzed by measuring the binding activity in total lysates to immobilized biotinylated hairpin oligonucleotides containing the indicated 18-bp targets. Assay were performed in duplicate. Binding site tested: Ap3, m12, m34, 8 other non-target oligos (a-h) and no oligo control (-).
- 25 Fig. 8 ELISA analysis of ZFPm2 DNA-binding specificity. Six-finger protein ZFPm2 was purified through affinity column from as MBP fusion protein from *E. coli*. Specificity of binding was analyzed by measuring the binding activity in total lysates to immobilized biotinylated hairpin oligonucleotides containing the indicated 18-bp targets. Assay were performed in duplicate. Binding site tested: Ap3, m12, m34, 8 other non-target oligos (a-h) and no oligo control (-).
- 30 Fig. 9 ELISA analysis of ZFPm3 DNA-binding specificity. Six-finger protein ZFPm3 was purified through affinity column from as MBP fusion protein from *E. coli*. Specificity of binding was analyzed by measuring the binding activity in total lysates to immobilized biotinylated hairpin oligonucleotides containing the indicated 18-bp targets. Assay were performed in duplicate. Binding site tested: Ap3, m12, m34, 8 other non-target oligos (a-h) and no oligo control (-).
- Fig. 10 ELISA analysis of ZFPm4 DNA-binding specificity. Six-finger protein ZFPm4 was purified through affinity column from as MBP fusion protein from *E. coli*. Specificity of binding was analyzed by measuring the binding activity in total lysates to immobilized biotinylated hairpin

oligonucleotides containing the indicated 18-bp targets. Assay were performed in duplicate. Binding site tested: Ap3, m12, m34, 8 other non-target oligos (a-h) and no oligo control (-).

- 5 Fig. 11 ELISA analysis of ZFPmAp3 DNA-binding specificity. Six-finger protein ZFPAp3 was purified through affinity column from as MBP fusion protein from *E. coli*. Specificity of binding was analyzed by measuring the binding activity in total lysates to immobilized biotinylated hairpin oligonucleotides containing the indicated 18-bp targets. Assay were performed in duplicate. Binding site tested: Ap3, m12, m34, 8 other non-target oligos (a-h) and no oligo control (-).
- 10 Fig. 12 Gel Shift analysis of ZFPm1 DNA-binding affinity. Six-finger protein ZFPm1 was purified through affinity column from as MBP fusion protein from *E. coli*. A. Affinity of binding was determined from a gel shift analysis of the binding of labeled m12 oligo to decreasing concentrations of the purified ZFPm1 protein. B. The affinity of ZFPm1 was calculated to be approximately 2nM from the concentration where one half of the labeled oligo is bound to the ZFPm1 protein.
- 15 Fig. 13 Gel Shift analysis of ZFPm2 DNA-binding affinity. Six-finger protein ZFPm2 was purified through affinity column from as MBP fusion protein from *E. coli*. Affinity of binding was determined from a gel shift analysis of the binding of labeled m12 oligo to decreasing concentrations of the purified ZFPm2 protein. The affinity of ZFPm2 was calculated to be approximately 7.5nM from the concentration where one half of the labeled oligo is bound to the ZFPm2 protein.
- 20 Fig. 14 Gel Shift analysis of ZFPm3 DNA-binding affinity. Six-finger protein ZFPm3 was purified through affinity column from as MBP fusion protein from *E. coli*. Affinity of binding was determined from a gel shift analysis of the binding of labeled m34 oligo to decreasing concentrations of the purified ZFPm3 protein. The affinity of ZFPm3 was calculated to be approximately 0.18nM from the concentration where one half of the labeled oligo is bound to the ZFPm3 protein.

- 5 Fig. 15 Gel Shift analysis of ZFPm4 DNA-binding affinity. Six-finger protein ZFPm4 was purified through affinity column from as MBP fusion protein from *E. coli*. Affinity of binding was determined from a gel shift analysis of the binding of labeled m34 oligo to decreasing concentrations of the purified ZFPm4 protein. The affinity of ZFPm4 was calculated to be approximately 0.25nM from the concentration where one half of the labeled oligo is bound to the ZFPm4 protein.
- 10 Fig. 16 Gel Shift analysis of ZFPAp3 DNA-binding affinity. Six-finger protein ZFPAp3 was purified through affinity column from as MBP fusion protein from *E. coli*. Affinity of binding was determined from a gel shift analysis of the binding of labeled Ap3 oligo to decreasing concentrations of the purified ZFPAp3 protein. The affinity of ZFPAp3 was calculated to be approximately 2.3nM from the concentration where one half of the labeled oligo is bound to the ZFPAp3 protein.
- 15 Fig. 17 *In vivo* characterization of newly synthesized zinc finger protein ZFPm1, ZFPm2, ZFPm3, and ZFPm4 in plant reporter system. The four activation fusion constructs are: ZmUbi::ZFPm1-VP64//nos, ZmUbi::ZFPm2-VP64//nos, ZmUbi::ZFPm3-VP64//nos, and ZmUbi::ZFPm4-VP64//nos. The reporter constructs are similar to reporter I in Fig. 1 except the 2C7 binding site was replaced by MIPS gene specific ZFP binding site m12 for ZFPm1 and ZFPm2 and by the second MIPS gene specific ZFP binding site m34 for ZFPm3 and ZFPm4.
- 20 Fig. 18 ZFP-Effector fusion constructs for *Arabidopsis* transformation. ZFP-Effector constructs for *Arabidopsis* transformation. A. Transient transformation vectors: pND3011 and pND3012 are transcriptional repressors. pND3014 and pND3013 are transcriptional activators. pND0001 is control vector. B. Stable transformation vectors for Agrobacteria mediated plant transformation method.
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- 5 Fig. 19 Transient activation of endogenous AP3 gene in *Arabidopsis* leaf cells. RT-PCR was used to detect AP3 expression in *Arabidopsis* leaf protoplasts transformed with a GFP (pND0001, control), m4-VP64 (pND3013, non-specific activation control), and Ap3-VP64 (pND3014, AP3 specific activation). When no RNA was used in the RT-PCR reaction, no bands were seen (-). Transient transformation of a GFP or m4-VP64 construct have no effect on AP3 expression. The AP3-specific activator Ap3-VP64 causes a clear activation of AP3 expression.
- 10 Fig. 20 Endogenous gene AP3 specifically repressed by the expression of ZFPAp3-repressor fusion protein in transgenic plant ND0052-2e. A. PCR identification of transgene ZFPAp3 in transgenic event ND0052-2e and wt plant. B. RT-PCR evaluation of endogenous gene AP3 expression level in transgenic event ND0052-2e and wild-type (wt) plant. In plant ND0052-2e, the expression of AP3 gene is significantly repressed by the expression of ZFPAp3-repressor (ZFPAp3-SID) fusion protein (Quantitative PCR shows a 46 fold of repression). The cDNA used in RT-PCR analysis was isolated from the flowers of each plant.
- 15 Fig. 21 Quantitative analysis of endogenous gene AP3 gene expression level in transgenic ZFPAp3 plants. The expression level of AP3 gene in transgenic plant is normalized to the wild type plant (defined as scale of 1). Repression event ND0052-2e shows a 46-fold of activation and ND0052-257 shows a 5-fold of repression. Activation event ND0052-1a shows a 2-fold of activation. Each sample was performed on triplicate assays.
- 20 Fig. 22 ZFP-Effector fusion constructs for maize transformation. A. MIPS gene specific activation constructs. B. MIPS gene specific repression constructs.
- 25 Fig. 23 Transient activation of endogenous MIPS gene in maize cells. Quantitative PCR was used to detect MIPS expression in maize cells transformed with a GFP (control), m1-VP64 (pND3015), m2-VP64
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(pND3023), m3-VP64 (pND3024), and m4-VP64 (pND3016). The activator ZFPm4-VP64 causes activation of MIPS expression.

Fig. 24 Nucleotide sequences of various nucleic acids and oligonucleotides disclosed in the present application.

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Modes for Carrying Out the Invention

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other databases referred to herein are incorporated by reference in their entirety. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in applications, published applications and other publications and sequences from GenBank and other data bases that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

As used herein, "a" or "an" means "at least one" or "one or more."

As used herein, "zinc finger protein, (zinc finger polypeptide, or ZFP)" refers to a polypeptide having nucleic acid, *e.g.*, DNA, binding domains that are stabilized by zinc.

The individual DNA binding domains are typically referred to as "fingers," such that a zinc finger protein or polypeptide has at least one finger, more typically two fingers, more preferably three fingers, or even more preferably four or five fingers, to at least six or more fingers. Each finger binds from two to four base pairs of DNA, typically three or four base pairs of DNA. A ZFP binds to a nucleic acid sequence called a target nucleic acid sequence. Each finger usually comprises an approximately 30 amino acids, zinc-chelating, DNA-binding subdomain. An exemplary motif of one class, the Cys2-His2 class (C2H2 motif), is -CYS-(X)2-4-CYS-(X)12-His-(X)3-5-His, where X is any amino acid, and a single zinc finger of this class consists of an alpha helix containing the two invariant histidine residues and the two cysteine residues of a single beta turn that binds a zinc cation (*see, e.g.*, Berg et al., *Science*, 271:1081-1085 (1996)). A zinc finger protein can have at least two DNA-binding domains, one of which is a zinc finger polypeptide,

linked to the other domain via a flexible linker. The two domains can be identical or different. Both domains can be zinc finger proteins, either identical or different zinc finger proteins. Alternatively, one domain can be a non-zinc finger DNA-binding protein, such as one from a transcription factor.

5 As used herein, "framework (or backbone) derived from a naturally occurring zinc finger protein" means that the protein or peptide sequence within the naturally occurring zinc finger protein that is involved in non-sequence specific binding with a target nucleotide sequence is not substantially changed from its natural sequence. For example, such framework (or backbone) derived from the naturally occurring zinc finger 10 protein maintains at least 50%, and preferably, 60%, 70%, 80%, 90%, 95%, 99% or 100% identity compared to its natural sequence in the non-sequence specific binding region. Alternatively, the nucleic acid encoding such framework (or backbone) derived from the naturally occurring zinc finger protein can be hybridizable with the nucleic acid encoding the naturally occurring zinc finger protein, either entirely or within the non-sequence specific binding region, under low, medium or high stringency condition. Preferably, the nucleic acid encoding such framework (or backbone) derived from the naturally occurring zinc finger protein is hybridizable with the nucleic acid encoding the naturally occurring zinc finger protein, either entirely or within the non-sequence specific binding region, under high stringency condition.

15 20 As used herein, "gene" refers to a nucleic acid molecule or portion thereof which comprises a coding sequence, optionally containing introns, and control regions which regulate the expression of the coding sequence and the transcription of untranslated portions of the transcript. Thus, the term "gene" includes, besides coding sequence, regulatory sequence such as the promoter, enhancer, 5' untranslated regions, 3' 25 untranslated region, termination signals, poly adenylation region and the like. Regulatory sequence of a gene may be located proximal to, within, or distal to the coding region.

As used herein, "target gene" refers to a gene whose expression is to be modulated by a zinc finger protein in plant cells.

30 As used herein, "plant" refers to any of various photosynthetic, eucaryotic multi-cellular organisms of the kingdom Plantae, characteristically producing embryos, containing chloroplasts, having cellulose cell walls and lacking locomotion. As used

herein, "plant" includes any plant or part of a plant at any stage of development, including seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, microspores, and progeny thereof.

5 Also included are cuttings, and cell or tissue cultures. As used in conjunction with the present invention, the term "plant tissue" includes, but is not limited to, whole plants, plant cells, plant organs, *e.g.*, leafs, stems, roots, meristems, plant seeds, protoplasts, callus, cell cultures, and any groups of plant cells organized into structural and/or functional units.

10 As used herein, "modulate the expression of a target gene in plant cells" refers to increasing (activation) or decreasing (repression) the expression of the target gene in plant cells with a zinc finger protein, alone or in combination with other transcription and/or translational regulatory factors, or nucleic acids encoding such zinc finger protein, in plant cells.

15 As used herein, "providing plant cells with a zinc finger protein" refers to the provisional to the plant cells, whether in culture or in whole plant, functional zinc finger protein that is capable of modulating a target gene in the plant cells. The functional zinc finger protein can be provided, *i.e.*, delivered, to the plant cells by any means. For example, the zinc finger protein can be delivered directly into the plant cells.

20 Alternatively and preferably, nucleic acids, *e.g.*, DNA or mRNA, encoding such zinc finger protein can be delivered into the plant cells and the plant cells are maintained under the conditions that functional zinc finger protein can be produced within the plant cells.

25 As used herein, a "target nucleotide sequence" refers to a portion of double-stranded polynucleotide acid, *e.g.*, RNA, DNA, PNA (peptide nucleic acid) or combinations thereof, to which it is advantageous to bind a protein. In one embodiment, a "target nucleotide sequence" is all or part of a transcriptional control element for a gene for which a desired phenotypic result can be attained by altering the degree of its expression. A transcriptional control element includes positive and negative control elements such as a promoter, an enhancer, other response elements, *e.g.*, steroid response element, heat shock response element, metal response element, a repressor binding site, operator, and/or a silencer. The transcriptional control element can be viral, eukaryotic,

or prokaryotic. A "target nucleotide sequence" also includes a downstream or an upstream sequence which can bind a protein and thereby modulate, typically prevent transcription.

As used herein, "specifically binds to a target nucleotide sequence" means that the binding affinity of a zinc finger protein to a specified target nucleic acid sequence is statistically higher than the binding affinity of the same zinc finger protein to a generally comparable, but non-target nucleic acid sequence, *e.g.*, a GNN sequence without matching code sequence for the particular zinc finger protein. Normally, the binding affinity of a zinc finger protein to a specified target nucleic acid sequence is at least 1.5 fold, and preferably 2 fold or 5 fold, of the binding affinity of the same zinc finger protein to a non-target nucleic acid sequence. It also refers to binding of a zinc-finger-protein-nucleic-acid-binding domain to a specified nucleic acid target sequence to a detectably greater degree, *e.g.*, at least 1.5-fold over background, than its binding to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. The zinc finger protein's Kd to each nucleotide sequence can be compared to assess the binding specificity of the zinc finger protein to a particular target nucleotide sequence.

As used herein, a "target nucleotide sequence within a target gene" refers to a functional relationship between the target nucleotide sequence and the target gene in that binding of a zinc-finger-protein to the target nucleotide sequence will modulate the expression of the target gene. The target nucleotide sequence can be physically located anywhere inside the boundaries of the target gene, *e.g.*, 5' ends, coding region, 3' ends, upstream and downstream regions outside of cDNA encoded region, or inside enhancer or other regulatory region, and can be proximal or distal to the target gene.

As used herein, "culturing plant cells" refers to the cultivation or growth of the plant cells. Such cultivation or growth can be *in vitro*, *e.g.*, in culture medium, or *in vivo*, *e.g.*, in planta.

As used herein, "endogenous" refers to nucleic acid or protein sequence naturally associated with a target gene or a host cell into which it is introduced.

As used herein, "exogenous" refers to nucleic acid or protein sequence not naturally associated with a target gene or a host cell into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring nucleic acid, *e.g.*, DNA

sequence, or naturally occurring nucleic acid sequence located in a non-naturally occurring genome location.

As used herein, "effector (or effector protein)" refer to constructs or their encoded products which are able to regulate gene expression either by activation or repression or which exert other effects on a target nucleic acid. The effector protein may include a zinc finger binding region only, but more commonly also includes a "functional domain" such as a "regulatory domain."

As used herein, "regulatory domain" refer to the portion of the effector protein or effector which enhances or represses gene expression.

As used herein, "compound" refer to any substance that is metabolized in plant cells and its metabolism can be modulated by a zinc-finger-protein in plant cells.

As used herein, "genetically modified plant (or transgenic plant)" refers to a plant which comprises within its genome an exogenous polynucleotide. Generally, and preferably, the exogenous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The exogenous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of exogenous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

As used herein, "essential gene" refers to a gene encoding a protein that is essential to the growth or survival of the plant, e.g., a biosynthetic enzyme, receptor, signal transduction protein, structural gene product, or transport protein.

As used herein, "expression cassette" refers to a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for

proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric,
5 meaning that at least one of its components is heterologous with respect to at least one of its other components. The zinc finger-effector fusions of the present invention are chimeric. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically,
10 however, the expression cassette is heterologous with respect to the host, *i.e.*, the particular DNA sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue or organ or stage of development. In the case of a plastid expression cassette, for expression of the nucleotide sequence from a plastid genome, additional elements, *i.e.*, ribosome binding sites, may be required.
15

As used herein, "minimal promoter" refers to a promoter element, particularly a TATA element, that is inactive or that has greatly reduced promoter activity in the absence of upstream activation. In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription.
20

As used herein, "significant increase" refer to an increase in gene expression, enzymatic or other biological activity, specificity or affinity, or effector or phenotypic activity, that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold or greater of the activity without the ZFP or ligand inducer, more preferably an increase by about 5-fold or greater, and most preferably an increase by about 10-fold or greater.
25

As used herein, "repressor protein" refer to a protein that binds to operator of DNA or to RNA to prevent transcription or translation, respectively.
30

As used herein, "repression" refer to inhibition of transcription or translation by binding of repressor protein to specific site on DNA or mRNA. Preferably, repression includes a significant change in transcription or translation level of at least 1.5 fold, more preferably at least two fold, and even more preferably at least five fold.

5 As used herein, "activator protein" refer to a protein that binds to operator of DNA or to RNA to enhance transcription or translation, respectively.

As used herein, "activation" refer to enhancement of transcription or translation by binding of activator protein to specific site on DNA or mRNA. Preferably, activation includes a significant change in transcription or translation level of at least 1.5 fold, more 10 preferably at least two fold, and even more preferably at least five fold.

As used herein, "conservatively modified variant" refer to amino acid and nucleic acid sequences containing individual substitutions, deletions or additions that alter, add or delete a single amino acid or nucleotide or a small percentage of amino acids or nucleotides in the sequence, where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants and alleles of the invention. The following groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Serine (S), Threonine (T); 3) Aspartic acid (D), Glutamic acid (E); 4) Asparagine (N), Glutamine (Q); 5) Cysteine (C), Methionine (M); 6) Arginine (R), Lysine (K), Histidine (H); 7) Isoleucine (I), Leucine (L), Valine (V); and 8) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). (see, e.g., Creighton, Proteins (1984) for a discussion of amino acid properties).

15 20 As used herein, a combination refers to any association between two or among more items.

As used herein, a composition refers to any mixture. It may be a solution, a suspension, liquid, powder, paste, aqueous, non-aqueous or any combination thereof.

As used herein, derivative or analog of a molecule refers to a portion derived from or a modified version of the molecule.

30 As used herein, operably linked, operatively linked or operationally associated refers to the functional relationship of DNA with regulatory and effector sequences of

nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. To optimize expression and/or *in vitro* transcription, it may be helpful to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, e.g., Kozak, *J. Biol. Chem.*, 266:19867-19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, a promoter region or promoter element refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

As used herein: stringency of hybridization in determining percentage mismatch is as follows: (1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C; (2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C; and (3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C. Equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

The term substantially identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

As used herein, isolated means that a substance is either present in a preparation at a concentration higher than that substance is found in nature or in its naturally occurring state or that the substance is present in a preparation that contains other materials with which the substance is not associated with in nature.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well known within the skill of the artisan. An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, "macromolecule" refers to a molecule that, without attaching to another molecule, is capable of generating an antibody that specifically binds to the macromolecule.

As used herein, "small molecule" refers to a molecule that, without forming homo-aggregates or without attaching to a macromolecule or adjuvant, is incapable of generating an antibody that specifically binds to the small molecule. Preferably, the small molecule has a molecular weight that is about or less than 10,000 daltons. More preferably, the small molecule has a molecular weight that is about or less than 5,000 dalton.

As used herein, "vitamin" refers to a trace organic substance required in certain biological species. Most vitamins function as components of certain coenzymes.

As used herein, "lipid" refers to water-insoluble, oily or greasy organic substances that are extractable from cells and tissues by nonpolar solvents, such as chloroform or ether.

As used herein, a "receptor" refers to a molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or synthetic molecules. Receptors may also be referred to in the art as anti-ligands. As used herein, the receptor and anti-ligand are interchangeable. Receptors can be used in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, or in physical contact with, to a binding member, either directly or indirectly via a specific binding substance or linker. Examples of receptors, include, but are not limited to: antibodies, cell membrane receptors surface receptors and internalizing receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants such as on viruses, cells, or other materials, drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles.

As used herein, "nutrient or storage protein" refers to a protein that is used by the cell as the nutrient source or storage form for such nutrient. Non-limiting examples of nutrient or storage proteins include gliadin, ovalbumin, casein, and ferritin.

As used herein, "contractile or motile protein" refers to a protein that endows cells and organisms with the ability to contract, to change shape, or to move about. Non-limiting examples of contractile or motile proteins include actin, myosin, tubulin and dynein.

As used herein, "structural protein" refers to a protein that serves as supporting filaments, cables, or sheets to give biological structures strength or protection. Non-

limiting examples of structural proteins include keratin, fibroin, collagen, elastin and proteoglycans.

As used herein, "defense protein" refers to a protein that defends organisms against invasion by other species or protect them from injury. Non-limiting examples of defense proteins include antibodies, fibrinogen, thrombin, botulinus toxin, diphtheria toxin, snake venoms and ricin.

As used herein, "regulatory protein" refers to a protein that helps regulate cellular or physiological activity. Non-limiting examples of regulatory proteins include insulin, growth hormones, corticotropin and repressors.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

B. Methods for modulating gene expression in plant

In one aspect, the present invention is directed to a method to modulate the expression of a target gene in plant cells, which method comprises providing plant cells with a zinc finger protein, said zinc finger protein being capable of binding or specifically binding to a target nucleotide sequence, or a complementary strand thereof, within a target gene, and allowing said zinc finger protein binding to said target nucleotide sequence, whereby the expression of said target gene in said plant cells is modulated.

Plant

The present invention can be used over a broad range of plant types, preferably the class of higher plants amenable to transformation techniques, particularly monocots and dicots. Particularly preferred are monocots such as the species of the Family Gramineae including Sorghum bicolor and Zea mays. The present method can also be used in species from the following genera: Cucurbita, Rosa, Vitis, Juglans, Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Ciahorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocallis, Nemesis, Pelargonium, Panieum, Pennisetum, Ranunculus, Senecio, Salpiglossis,

Cucumis, Browalia, Glycine, Pisum, Phaseolus, Lolium, Oryza, Avena, Hordeum, Secale, and Triticum.

Preferred plant cells include those from corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), particularly those *Brassica* species useful as sources of seed oil, 5 alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), duckweed (*Lemna*) soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), 10 potato (*Solanum tuberosum*), peanut (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea spp.*), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus tree (*Citrus spp.*), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa spp.*), avocado (*Persea americana*), fig (*Ficus carica*), guava (*Psidium guajava*), mango 15 (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beet (*Beta vulgaris*), sugarcane (*Saccharum spp.*), oats, barley, vegetables, ornamentals, and conifers.

Preferred vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus spp.*), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*).

Preferred ornamentals include azalea (*Rhododendron spp.*), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosa-sinensis*), roses (*Rosa spp.*), tulips (25 *Tulipa spp.*), daffodils (*Narcissus spp.*), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing the present method include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliottii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies* 30

amabilis) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).

Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden bean, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc. Legumes include, but are not limited to, *Arachis*, e.g., peanuts, *Vicia*, e.g., crown vetch, hairy vetch, adzuki bean, mung bean, and chickpea, *Lupinus*, e.g., lupine, trifolium, *Phaseolus*, e.g., common bean and lima bean, *Pisum*, e.g., field bean, *Melilotus*, e.g., clover, *Medicago*, e.g., alfalfa, *Lotus*, e.g., trefoil, lens, e.g., lentil, and false indigo. Preferred forage and turf grass for use in the methods of the invention include alfalfa, orchard grass, tall fescue, perennial ryegrass, creeping bent grass, and redtop.

More preferably, plants that can be modulated by the present method are crop plants and model plant, e.g., corn, rice, alfalfa, sunflower, canola, soybean, cotton, peanut, sorghum, wheat, tobacco, lemna, etc. Most preferably, gene expression in corn, rice, lemna, and soybean plants are modulated by the present method.

Zinc finger proteins used in the present method

Any zinc finger proteins can be used in the present method. In addition, zinc finger proteins disclosed or designed and predicted according to the procedures in WO 98/54311 can be used. WO 98/54311 discloses technology which allows the design of zinc finger protein domains which bind specific nucleotide sequences which are unique to a target gene. It has been calculated that a sequence comprising 18 nucleotides is sufficient to specify an unique location in the genome of higher organisms. Typically, therefore, the zinc finger protein domains are hexadactyl, i.e., contain 6 zinc fingers, each with its specifically designed alpha helix for interaction with a particular triplet. However, in some instances, a shorter or longer nucleotide target sequence may be desirable. Thus, the zinc finger domains in the proteins may contain from 2-12 fingers, preferably, 3-8 fingers, more preferably 5-7 fingers, and most preferably 6 fingers.

When a multi-finger protein binds to a polynucleotide duplex, e.g., DNA, RNA, PNA or any hybrids thereof, its fingers typically line up along the polynucleotide duplex with a periodicity of about one finger per 3 bases of nucleotide sequence. The binding

sites of individual zinc fingers (or subsites) typically span three to four bases, and
subsites of adjacent fingers usually overlap by one base. Accordingly, a three-finger zinc
finger protein XYZ binds to the 10 base pair site abcdefghij (wherein each "X" indicates
one of the duplex DNA) with the subsite of finger X being ghij, finger Y being defg and
finger Z being abcd. For example, as known in the art, to design a three-finger zinc
finger protein to bind to the targeted 10 base site abcdefXXXX (wherein each "X"
represents a base that would be specified in a particular application), zinc fingers Y and Z
would have the same polypeptide sequence as found in the original zinc finger discussed
above (perhaps a wild type zinc fingers which bind defg and abcd, respectively). Finger
X would have a mutated polypeptide sequence. Preferably, finger X would have
mutations at one or more of the base-contacting positions, i.e., finger X would have the
same polypeptide sequence as a wild type zinc finger except that at least one of the four
amino residues at the primary positions would differ. Similarly, to design a three-finger
zinc protein that would bind to a 10 base sequence abcXXXXhij (wherein each "X" is
base that would be specified in a particular application), fingers X and Z have the same
sequence as the wild type zinc fingers which bind ghij and abcd, respectively, while
finger Y would have residues at one or more base-coating positions which differ from
those in a wild type finger. The present method can employ multi-fingered proteins in
which more than one finger differs from a wild type zinc finger. The present method can
also employ multi-fingered protein in which the amino acid sequence in all the fingers
have been changed, including those designed by combinatorial chemistry or other protein
design and binding assays.

It is also possible to design or select a zinc finger protein to bind to a targeted
polynucleotide in which more than four bases have been altered. In this case, more than
one finger of the binding protein must be altered. For example, in the 10 base sequence
XXXdefgXXX, a three-finger binding protein could be designed in which fingers X and
Z differ from the corresponding fingers in a wild type zinc finger, while finger Y will
have the same polypeptide sequence as the corresponding finger in the wild type fingers
which binds to the subsite defg. Binding proteins having more than three fingers can also
be designed for base sequences of longer length. For example, a four finger-protein will
optimally bind to a 13 base sequence, while a five-finger protein will optimally bind to a

16 base sequence. A multi-finger protein can also be designed in which some of the fingers are not involved in binding to the selected DNA. Slight variations are also possible in the spacing of the fingers and framework.

5 While the present method can employ any valid recognition code, the zinc finger protein nucleic acid binding domain/nucleoside binding partner pairs disclosed in United States Patent No. 5,789,538, WO 96/06166, and WO 00/23464 can also be used.

10 Methods for designing and identifying a zinc finger protein with desired nucleic acid binding characteristics also include those described in WO98/53060, which reports a method for preparing a nucleic acid binding protein of the Cys2-His2 zinc finger class capable of binding to a nucleic acid quadruplet in a target nucleic acid sequence.

15 Zinc finger proteins useful in the present method can comprise at least one zinc finger polypeptide linked via a linker, preferably a flexible linker, to at least a second DNA binding domain, which optionally is a second zinc finger polypeptide. The zinc finger protein may contain more than two DNA-binding domains, as well as one or more regulator domains. The zinc finger polypeptides used in the present method can be engineered to recognize a selected target site in the gene of choice. Typically, a backbone from any suitable C2H2-ZFP, such as SPA, SPIC, or ZIF268, is used as the scaffold for the engineered zinc finger polypeptides (*see, e.g.*, Jacobs, *EMBO J.* (1992) 11:4507 ; and Desjarlais & Berg, *Proc. Natl. Acad. Sci. USA* (1993) 90:2256-2260). A number of methods can then be used to design and select a zinc finger polypeptide with high affinity for its target. A zinc finger polypeptide can be designed or selected to bind to any suitable target site in the target gene, with high affinity.

20 Any suitable method known in the art can be used to design and construct nucleic acids encoding zinc finger polypeptides, *e.g.*, phage display, random mutagenesis, combinatorial libraries, computer/rational design, affinity selection, PCR, cloning from cDNA or genomic libraries, synthetic construction and the like. (*see, e.g.*, U.S. Pat. No. 5,786,538; Wu *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:344-348; Jamieson *et al.*, *Biochemistl.* (1994) 33:5689-5695; Rebar & Pabo, *Science* (1994) 263:671-673; Choo & Klug, *Proc. Natl. Acad. Sci. USA* (1994) 91: 11168-11172; Pomerantz *et al.*, *Science*, 25 267:93-96 (1995); Pomerantz *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:9752-9756;

Liu *et al.*, *Proc. Natl. Acad. Sci. USA* (1997) 94:5525-5530; and Desjarlais & Berg, (1994) *Proc. Natl. Acad. Sci. USA* 91:11-99-11103).

Zinc finger proteins useful in the method can be made by any recombinant DNA technology method for gene construction. For example, PCR based construction can be used. Ligation of desired fragments can also be performed, using linkers or appropriately complementary restriction sites. One can also synthesize entire finger domain or parts thereof by any protein synthesis method. Preferred for cost and flexibility is the use of PCR primers that encode a finger sequence or part thereof with known base pair specificity, and that can be reused or recombined to create new combinations of fingers and ZFP sequences.

The amino acid linker should be flexible, a beta turn structure is preferred, to allow each three finger domain to independently bind to its target sequence and avoid steric hindrance of each other's binding. Linkers can be designed and empirically tested.

If recognition code is incomplete, or if desired, in one embodiment, the ZFP can be designed to bind to non-contiguous target sequences. For example, a target sequence for a six-finger ZFP can be a nine base pair sequence (recognized by three fingers) with intervening bases (that do not contact the zinc finger nucleic acid binding domain) between a second nine base pair sequence (recognized by a second set of three fingers). The number of intervening bases can vary, such that one can compensate for this intervening distance with an appropriately designed amino acid linker between the two three-finger parts of ZFP. A range of intervening nucleic acid bases in a target binding site is preferably 20 or less bases, more preferably 10 or less, and even more preferably 6 or less bases. It is of course recognized that the linker must maintain the reading frame between the linked parts of ZFP protein.

A minimum length of a linker is the length that would allow the two zinc finger domains to be connected without providing steric hindrance to the domains or the linker. A linker that provides more than the minimum length is a "flexible linker." Determining the length of minimum linkers and flexible linkers can be performed using physical or computer models of DNA-binding proteins bound to their respective target sites as are known in the art.

The six-finger zinc finger peptides can use a conventional "TGEKP" linker to connect two three-finger zinc finger peptides or to add additional fingers to a three-finger protein. Other zinc finger peptide linkers, both natural and synthetic, are also suitable.

A useful zinc finger framework is that of ZIF268 (see WO00/23464 and references cited therein.), however, others are suitable. Examples of known zinc finger nucleotide binding polypeptides that can be truncated, expanded, and/or mutagenized in order to change the function of a nucleotide sequence containing a zinc finger nucleotide binding motif includes TFIIIA and zif268. Other zinc finger nucleotide binding proteins are known to those of skill in the art. The murine CYS2-His2 zinc finger protein Zif268 is structurally well characterized of the zinc finger proteins (Pavletich and Pabo, *Science* (1991) 252:809-817; Elrod-Erickson *et al.*, *Structure (London)* (1996) 4:1171-1180; and Swirnoff *et al.*, *Mol. Cell. Biol.* (1995) 15:2275-2287). DNA recognition in each of the three zinc finger domains of this protein is mediated by residues in the N-terminus of the alpha-helix contacting primarily three nucleotides on a single strand of the DNA. The operator binding site for this three finger protein is 5'-GCGTGGGCG-3'. Structural studies of Zif268 and other related zinc finger-DNA complexes (Elrod-Erickson *et al.*, *Structure (London)* (1998) 6:451-464; Kim and Berg, *Nature Structural Biology* (1996) 3:940-945; Pavletich and Pabo, *Science* (1993) 261:1701-1707; Houbaviy *et al.*, *Proc. Natl. Acad. Sci. USA* (1996) 93:13577-13582; Fairall *et al.*, *Nature (London)* (1993) 366:483-487; Wuttke *et al.*, *J. Mol. Biol.* (1997) 273:183-206; Nolte *et al.*, *Proc. Natl. Acad. Sci. USA* (1998) 95:2938-2943; and Narayan *et al.*, *J. Biol. Chem.* (1997) 272:7801-7809) have shown that residues from primarily three positions on the α -helix, -1, 3, and 6, are involved in specific base contacts. Typically, the residue at position -1 of the α -helix contacts the 3' base of that finger's subsite while positions 3 and 6 contact the middle base and the 5' base, respectively.

However, it should be noted that at least in some cases, zinc finger domains appear to specify overlapping 4 bp sites rather than individual 3 bp sites. In Zif268, residues in addition to those found at helix positions -1, 3, and 6 are involved in contacting DNA (Elrod-Erickson *et al.*, *Structure* (1996) 4:1171-1180). Specifically, an aspartate in helix position 2 of the middle finger plays several roles in recognition and makes a variety of contacts. The carboxylate of the aspartate side chain hydrogen bonds

with arginine at position -1, stabilizing its interaction with the 3'- guanine of its target site. This aspartate may also participate in water-mediated contacts with the guanine's complementary cytosine. In addition, this carboxylate is observed to make a direct contact to the N4 of the cytosine base on the opposite strand of the 5'-guanine base of the finger 1 binding site. It is this interaction which is the chemical basis for target site overlap.

Any suitable method of protein purification known to those of skill in the art can be used to purify the zinc finger proteins of the invention (see Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York) (1989)). In addition, any suitable host can be used, *e.g.*, bacterial cells, insect cells, yeast cells, mammalian cells, and the like.

In one embodiment, expression of the zinc finger protein fused to a maltose binding protein (MBP-zinc finger protein) in bacterial strain JM 109 allows for purification through an amylose column (NEB). High expression levels of the zinc finger protein can be obtained by induction with IPTG since the MBP-zinc finger protein fusion in the pMal-c2 expression plasmid is under the control of the IPTG inducible tac promoter (NEB). Bacteria containing the MBP-zinc finger protein fusion plasmids are inoculated in to 2xYT medium containing 10~LM ZnC12, 0.02% glucose, plus 50 ptg/ml ampicillin and shaken at 37°C. At mid-exponential growth IPTG is added to 0.3 mM and the cultures are allowed to shake. After 3 hours the bacteria are harvested by centrifugation, disrupted by sonication, and then insoluble material is removed by centrifugation. The MBP-zinc finger proteins are captured on an amylose-bound resin, washed extensively with buffer containing 20 mM Tris-HCl (pH 7-5), 200 mM NaCl, 5 mM DTT and 50 ~tM ZnC12, then eluted with maltose in essentially the same buffer (purification is based on a standard protocol from NEB). Purified proteins are quantitated and stored for biochemical analysis.

The biochemical properties of the purified proteins, *e.g.*, Kd, can be characterized by any suitable assay. In one embodiment, Kd is characterized via electrophoretic mobility shift assays ("EMSA") (Buratowski & Chodosh, in Current Protocols in Molecular Biology pp. 12.2.1-12.2.7 (Ausubel ed., 1996)). By "Kd" is meant the dissociation constant for the compound, *i.e.*, the concentration of a compound (*e.g.*, a zinc

finger protein) that gives half maximal binding of the compound to its target (*i.e.*, half of the compound molecules are bound to the target) under given conditions (*i.e.*, when [target] << Kd), as measured using a given assay system. Any assay system can be used, as long as it gives an accurate measurement of the actual Kd of the zinc finger protein. In 5 one embodiment, the Kd for the zinc finger proteins of the invention is measured using an electrophoretic mobility shift assay as described herein.

As described in the Example Section below, the observed affinities of 6-finger proteins tested were determined to be: ZFPm1=2.0nM, ZFPm2=7.5 nM, ZFPm3= 0.18 nM, ZFPm4=0.25 nM and ZFPAp3=2.3 nM (Fig. 12-16). Most of these affinities 10 compare favorably with existing 6 finger proteins. The 2C7 derivative of the Sp1 zinc finger was reported to have a specificity of 0.46 nM and the e2c zinc finger was reported to have an affinity of 0.5 nM. Consequently, zinc fingers used in the invention typically have affinity 0.1 to 1.0 nanomolar range, more typically 0.18 to 0.75 nanomolar, and to picomolar range and even to femtomolar range. Typically, naturally occurring zinc 15 fingers have Kd in the nanomolar range. Zinc finger proteins useful in the invention may even have an affinity for the target site that is in the femtamolar range, *e.g.*, 100 femtamoles, 10 femtamoles, or less, in some cases as low as one femtamole.

In a specific embodiment, the zinc finger protein used in the present methods comprises a framework (or backbone) derived from a naturally occurring zinc finger protein. Framework (or backbone) derived from any naturally occurring zinc finger protein can be used. For example, the zinc finger protein comprises a framework (or backbone) derived from a zinc finger protein comprising a C2H2 motif can be used. Preferably, the protein or peptide sequence within the β sheet of the C2H2 motif is not 20 substantially changed, or not changed, from its natural sequence.

25 In another specific embodiment, the zinc finger protein used in the present methods comprises a framework (or backbone) derived from a zinc finger protein that is naturally functional in plant cells. For example, the zinc finger protein used in the present methods can comprise a C3H zinc finger (Terol et al., *Gene*, 260(1-2):45-53 (2000)), a QALGGH motif (Takatsuji, *Plant. Mol. Biol.*, 39(6):1073-8 (1999)), a RING- 30 H2 zinc finger motif (Jensen et al., *FEBS Lett.*, 436(2):283-7 (1998)), a 9 amino acid C2H2 motif (Chou et al., *Proc. Natl. Acad. Sci. USA*, 95(9):5293-8 (1998)), a zinc finger

motif of Arabidopsis LSD1 (Dietrich et al., *Cell*, 88(5):685-94 (1997)) and a zinc finger motif of BBF/Dof domain proteins (De Paolis et al., *Plant J.*, 10(2):215-23 (1996)).

In another specific embodiment, the zinc finger protein used in the present methods comprises a framework (or backbone) derived from a zinc finger protein that is known in the art as of January 19, 2001.

For example, the zinc finger protein used in the present methods can comprise a framework (or backbone) derived from the zinc finger protein disclosed in the following U.S. patents and PCT patent publications: U.S. Patent No. 6,160,091, 6,140,466, 6,140,081, 5,831,008, 5,811,304 and 5,789,538 and WO 00/42219, WO 00/41566, WO 00/27878, WO 00/23464, WO 00/20622, WO 00/20556, WO 99/62952, WO 99/48909, WO 99/46293, WO 99/45132, WO 99/42474, WO 99/21991, WO 98/54311, WO 98/53061, WO 98/45326, WO 96/11267, WO 96/06168 and WO 95/19431.

The zinc finger protein used in the present methods can also comprise a framework (or backbone) derived from the zinc finger protein disclosed in the following references: 1) Cousins RJ, Lanningham-Foster L., "Regulation of cysteine-rich intestinal protein, a zinc finger protein, by mediators of the immune response." *J Infect Dis.* 2000 Sep;182 Suppl 1:S81-4; 2) Bouhouche N, Syvanen M, Kado CI., "The origin of prokaryotic C2H2 zinc finger regulators." *Trends Microbiol.* 2000 Feb;8(2):77-81; 3: Klug A., "Zinc finger peptides for the regulation of gene expression." *J Mol Biol.* 1999 Oct 22;293(2):215-8; 4) Cook T, Gebelein B, Urrutia R., "Sp1 and its likes: biochemical and functional predictions for a growing family of zinc finger transcription factors." *Ann N Y Acad Sci.* 1999 Jun 30;880:94-102; 5) Takatsuji H., "Zinc-finger proteins: the classical zinc finger emerges in contemporary plant science." *Plant Mol Biol.* 1999 Apr;39(6):1073-8; 6) Huang S., "The retinoblastoma protein-interacting zinc finger gene RIZ in 1p36-linked cancers." *Front Biosci.* 1999 Jun 15;4:D528-32; 7: Krempler A, Brenig B., "Zinc finger proteins: watchdogs in muscle development." *Mol Gen Genet.* 1999 Mar;261(2):209-15; 8) Hoffmann A, Ciani E, Houssami S, Brabet P, Journot L, Spengler D., "Induction of type I PACAP receptor expression by the new zinc finger protein Zac1 and p53." *Ann N Y Acad Sci.* 1998 Dec 11;865:49-58; 9) Kroncke KD, Kolb-Bachofen V., "Measurement of nitric oxide-mediated effects on zinc homeostasis and zinc finger transcription factors." *Methods Enzymol.* 1999;301:126-35; 10) Tseng H.,

“Basonuclin, a zinc finger protein associated with epithelial expansion and proliferation.” Front Biosci. 1998 Sep 1;3:D985-8; 11) Takatsuji H., “Zinc-finger transcription factors in plants.” Cell Mol Life Sci. 1998 Jun;54(6):582-96; 12) Chilton BS, Hewetson A., “Zinc finger proteins RUSH in where others fear to tread.” Biol Reprod. 1998 Feb;58(2):285-94; 13) Urrutia R., “Exploring the role of homeobox and zinc finger proteins in pancreatic cell proliferation, differentiation, and apoptosis.” Int J Pancreatol. 1997 Aug;22(1):1-14; 14) Mizuno K, Higuchi O., “LIM domains: double zinc finger motifs involved in protein-protein interactions” Tanpakushitsu Kakusan Koso. 1997 Oct;42(13):2061-71; 15: Losson R., “KRAB zinc finger proteins and nuclear receptors: a possible cross-talk.” Biol Chem. 1997 Jul;378(7):579-81; 16) Eis PS., “Fluorescence studies of zinc finger peptides and proteins.” Methods Enzymol. 1997;278:330-43; 17) Tamaoki T, Hashimoto T., “ZFH/ATBF1 gene family: transcription factors containing both homeo- and zinc finger-domains” Tanpakushitsu Kakusan Koso. 1996 Sep;41(11):1550-9; 18: Hollemann T, Bellefroid E, Stick R, Pieler T., “Zinc finger proteins in early Xenopus development.” Int J Dev Biol. 1996 Feb;40(1):291-5; 19) Kroncke KD, Kolb-Bachofen V., “Detection of nitric oxide interaction with zinc finger proteins.” Methods Enzymol. 1996;269:279-84; 20) Lopingco MC, Perkins AS., “Molecular analysis of Evi1, a zinc finger oncogene involved in myeloid leukemia.” Curr Top Microbiol Immunol. 1996;211:211-22: 21) Hromas R, Davis B, Rauscher FJ 3rd, Klemsz M, Tenen D, Hoffman S, Xu D, Morris JF., “Hematopoietic transcriptional regulation by the myeloid zinc finger gene, MZF-1.” Curr Top Microbiol Immunol. 1996;211:159-64; 22) Nagaoka M, Sugiura Y., Conversion of zinc finger protein to artificial site-specific nuclease: application to chromosome mapping and sequencing” Nippon Rinsho. 1995 Oct;53(10):2589-96; 23) Sarkar B. “Metal replacement in DNA-binding zinc finger proteins and its relevance to mutagenicity and carcinogenicity through free radical generation.” Nutrition. 1995 Sep-Oct;11(5 Suppl):646-9; 24) Gashler A, Sukhatme VP., “Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors.” Prog Nucleic Acid Res Mol Biol. 1995;50:191-224; 25) Knegtel RM, van Tilborg MA, Boelens R, Kaptein R., ‘NMR structural studies on the zinc finger domains of nuclear hormone receptors.” EXS. 1995;73:279-95; 26) Sanchez-Garcia I, Rabbitts TH., “The LIM domain: a new structural motif found in zinc-finger-

like proteins." Trends Genet. 1994 Sep;10(9):315-20; 27) Pieler T, Bellefroid E., "Perspectives on zinc finger protein function and evolution--an update." Mol Biol Rep. 1994 Jul;20(1):1-8; 28) Madden SL, Rauscher FJ 3d., "Positive and negative regulation of transcription and cell growth mediated by the EGR family of zinc-finger gene products." Ann N Y Acad Sci. 1993 Jun 11;684:75-84; 29) Thiesen HJ, Bach C., "DNA recognition of C2H2 zinc-finger proteins. Evidence for a zinc-finger-specific DNA recognition code." Ann N Y Acad Sci. 1993 Jun 11;684:246-9; 30) Freemont PS., "The RING finger. A novel protein sequence motif related to the zinc finger." Ann N Y Acad Sci. 1993 Jun 11;684:174-92; 31) Werner H, Roberts CT Jr, LeRoith D., "The regulation of IGF-I receptor gene expression by positive and negative zinc-finger transcription factors." Adv Exp Med Biol. 1993;343:91-103; 32) Berg JM., "Sp1 and the subfamily of zinc finger proteins with guanine-rich binding sites." Proc Natl Acad Sci U S A. 1992 Dec 1;89(23):11109-10; 33) Reddy BA, Etkin LD, Freemont PS., "A novel zinc finger coiled-coil domain in a family of nuclear proteins." Trends Biochem Sci. 1992 Sep;17(9):344-5; 34) Freedman LP., "Anatomy of the steroid receptor zinc finger region." Endocr Rev. 1992 May;13(2):129-45; 35) el-Baradi T, Pieler T., "Zinc finger proteins: what we know and what we would like to know." Mech Dev. 1991 Nov;35(3):155-69; 36) Schule R, Evans RM., "Cross-coupling of signal transduction pathways: zinc finger meets leucine zipper." Trends Genet. 1991 Nov-Dec;7(11-12):377-81; 37) Summers MF., Zinc finger motif for single-stranded nucleic acids? Investigations by nuclear magnetic resonance." J Cell Biochem. 1991 Jan;45(1):41-8; 38) Ashworth A, Denny P., "Zinc finger protein genes in the mouse genome." Mamm Genome. 1991;1(3):196-200; 39) South TL, Kim B, Hare DR, Summers MF., "Zinc fingers and molecular recognition. Structure and nucleic acid binding studies of an HIV zinc finger-like domain." Biochem Pharmacol. 1990 Jul 1;40(1):123-9; 40) Berg JM., "Zinc finger domains: hypotheses and current knowledge." Annu Rev Biophys Biophys Chem. 1990;19:405-21; 41) Copeland NG, Jenkins NA., "Retroviral integration in murine myeloid tumors to identify Evi-1, a novel locus encoding a zinc-finger protein." Adv Cancer Res. 1990;54:141-57; 42) Erickson RP, Verga V., "Is zinc-finger Y the sex-determining gene?" Am J Hum Genet. 1989 Nov;45(5):671-4; and 43) Struhl K., "Helix-

turn-helix, zinc-finger, and leucine-zipper motifs for eukaryotic transcriptional regulatory proteins." Trends Biochem Sci. 1989 Apr;14(4):137-40.

Effector domains

5 Although in some cases, a zinc finger protein itself is sufficient for modulating gene expression, the zinc finger protein is preferably fused to an effector domain (or regulatory domain or functional domain), *i.e.*, a protein domain which activates or represses gene expression, *e.g.*, an activator domain of a regulatory protein or an active domain of a nucleic acid modifying protein. The foregoing terms - *i.e.*, "effector domain 10 (or effector)," "regulatory domain," and "functional domain" may refer to the materials on either the nucleic acid or protein level as will be clear from the context.

15 The effector domain can have an activity such as transcriptional modulation activity, DNA modifying activity, protein modifying activity and the like when tethered to a DNA binding domain, *i.e.*, a zinc finger protein. Examples of regulatory domains include proteins or effector domains of proteins such as transcription factors and co-factors, *e.g.*, KRAB, MAD, ERD, SID, nuclear factor kappa B subunit p65, early growth response factor 1, and nuclear hormone receptors, VP16 and VP64, endonucleases, integrases, recombinases, methyltransferases, histone acetyltransferases, histone deacetylases, mutases, restriction enzymes, etc.

20 Activators and repressors include co-activators and co-repressors (*see, e.g.*, Utley *et al.*, *Nature* (1998) 394:498-502; and WO 00/03026). Effector domains can include DNA-binding domains from a protein that is not a zinc finger protein, such as a restriction enzyme, a nuclear hormone receptor, a homeodomain protein such as engrailed or antenopedia, a bacterial helix-turn-helix motif protein such as lambda 25 repressor and tet repressor, Gal4, TATA binding protein, helix-loop-helix motif proteins such as myc and myo D, leucine zipper type proteins such as fos and jun, and beta sheet motif proteins such as met, arc, and mnt repressors. Particularly preferred activator is the C1 activator domain of maize (Goff *et al.*, *Genes Dev.* (1991) 5(2):298-309).

30 In a specific embodiment, the zinc finger protein having an effector domain is one that is responsive to a ligand. The effector domain can effect such a response. Use of ligand inducible binding domain-effector fusions is generally known as a gene switch.

Therefore, the ZFP domains discussed here can be used as part of the ligand-binding domain in gene switches. Examples of such ligand-responsive domains include hormone receptor ligand binding domains, e.g., estrogen receptor domain, ecdysone receptor system, glucocorticosteroid (Parker, *Curr. Opin. Cell Biol.* (1993) 5:499-504; Beato *et al.*, *Cell* (1995) 83:851-857; Mangelsdorf *et al.*, *Cell* (1995) 83:835-839; and Yamamoto, *Annu. Rev. Genet.* (1985) 19:209-252), inducible promoters of trehalose biosynthetic genes (WO 99/46370), chemically inducible arabidopsis PR-1 promoter (WO 98/03536; and U.S. Patent No. 5,689,044) and a chemically inducible promoter of a cucumber chitinase/lysozyme gene (U.S. Patent No. 5,654,414). Preferred inducers are small, inorganic, biodegradable, molecules.

The zinc finger protein can be covalently or non-covalently associated with one or more regulatory domains. Alternatively, two or more regulatory domains, whether identical or different ones, can be linked together. The regulatory domains can be covalently linked to the zinc finger protein nucleic acid binding domain, e.g., via an amino acid linker, as part of a fusion protein. The zinc finger proteins can also be associated with a regulatory domain via a non-covalent dimerization domain, e.g., a leucine zipper, a STAT protein N terminal domain, or an FK506 binding protein (see, e.g., O'Shea, *Science* (1991) 254:539; Barahmand-Pour *et al.*, *Curr. Top. Microbiol. Immunol.* (1996) 211:121-128; Klemm *et al.*, *Annu. Rev. Immunol.* (1998) 16:569-592; Klemm *et al.*, *Annu. Rev. Immunol.* (1998) 16:569-592; Ho *et al.*, *Nature* (1996) 382:822-826; and Pomeranz *et al.*, *Biochem.* (1998) 37:965). The regulatory domain can be associated with the zinc finger protein domain at any suitable position, including the C- or N-terminus of the zinc finger protein.

Common regulatory domains for addition to the zinc finger protein made using the methods of the invention include, e.g., DNA-binding domains from transcription factors, effector domains from transcription factors (activators, repressors, co-activators, co-repressors), silencers, nuclear hormone receptors, and chromatin associated proteins and their modifiers, e.g., methylases, kinases, acetylases and deacetylases.

Transcription factor polypeptides from which one can obtain a regulatory domain include those that are involved in regulated and basal transcription. Such polypeptides include transcription factors, their effector domains, coactivators, silencers, nuclear

hormone receptors (*see, e.g.*, Goodrich *et al.*, *Cell* (1996) 84:825-830) for a review of proteins and nucleic acid elements involved in transcription. Transcription factors in general are reviewed in Bames and Adcock, *Clin. Exp. Allerg* v 25 Suppl. 2:46-49 (1995) and Roeder, *Methods Enz.* (1996) 273:165-171. Databases dedicated to transcription factors are also known (*see, e.g.*, Williams, *Science* (1995) 269:630). Nuclear hormone receptor transcription factors are described in, for example, Rosen *et al.*, *J. Med. Chem.* (1995) 38:4855-4874. The C/EBP family of transcription factors are reviewed in Wedel *et al.*, *Immunobiology* (1995) 193:171-185. Coactivators and co-repressors that mediate transcription regulation by nuclear hormone receptors are reviewed in, for example, Meier, *Eur. J. Endocrinol.* (1996) 134(2):158-9; Kaiser *et al.*, *Trends Biochem. Sci.* (1996) 21:342-345; and Utley *et al.*, *Nature* (1998) 394:498-502. GATA transcription factors, which are involved in regulation of hematopoiesis, are described in, for example, Simon, *Nat. Genet.* (1995) 11:9-11; and Weiss *et al.*, *Exp. Hematol.* 23:99-107. TATA box binding protein (T13P) and its associated TAF polypeptides (which include TAF30, TAF55, TAF80, TAF110, TAF150, and TAF250) are described in Goodrich & Tjian, *Curr. Opin. Cell Biol.* (1994) 6:403-409 and Hurley, *Curr. Opin. Struct. Biol.* (1996) 6:69-75. The STAT family of transcription factors are reviewed in, for example, Barahmand-Pour *et al.*, *Curr. Top. Microbiol. Immunol.* (1996) 211:121-128. Transcription factors involved in disease are reviewed in Aso *et al.*, *J Clin. Invest.* (1996) 97:1561-1569.

In one embodiment, the KRAB repression domain from the human KOX-I protein is used as a transcriptional repressor (Thiesen *et al.*, *New Biologist* (1990) 2:363-374; Margolin *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91:4509-4513; Pengue *et al.*, *Nucl. Acids Res.* (1994) 22:2908-2914; and Witzgall *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91:4514-4518). In another embodiment, KAP-1, a KRAB co-repressor, is used with KRAB (Friedman *et al.*, *Genes Dev.* (1996) 10:2067-2078). Alternatively, KAP-1 can be used alone with a zinc finger protein. Other preferred transcription factors and transcription factor domains that act as transcriptional repressors include MAD (*see, e.g.*, Sommer *et al.*, *J Biol. Chem.* (1998) 273:6632-6642; Gupta *et al.*, *Oncogene* (1998) 16:1149-1159; Queva *et al.*, *Oncogene* (1998) 16:967-977; Larsson *et al.*, *Oncogene* (1997) :737-748; Laherty *et al.*, *Cell* (1997) 89:349-356; and Cultraro *et al.*, *Mol Cell.*

Biol. (1997) 17:2353-2359); FKHR (forkhead in rhabdosarcoma gene) (Ginsberg *et al.*, *Cancer Res.* (1998) 15:3542-3546; and Epstein *et al.*, *Mol. Cell. Biol.* (1998) 18:4118-4130); EGR- I (early growth response gene product- 1) (Yan *et al.*, *Proc. Natl. Acad. Sci. USA* (1998) 95:8298-8303; and Liu *et al.*, *Cancer Gene Ther.* (1998) 5:3-28); the ets2 5 repressor factor repressor domain (ERD) (Sgouras *et al.*, *EMBO J* (1995) 14:4781-4793); and the MAD smSIN3 interaction domain (SID) (Ayer *et al.*, *Mol. Cell. Biol.* (1996) 16:5772-5781).

In another embodiment, the HSV VP 16 activation domain is used as a transcriptional activator (*see, e.g.*, Hagmann *et al.*, *J Virol.* (1997) 71:5952- 5962). Other preferred transcription factors that could supply activation domains include the VP64 activation domain (Selpel *et al.*, *EMBO J* (1996) 11:4961-4968); nuclear hormone receptors (*see, e.g.*, Torchia *et al.*, *Curr. Opin. Cell. Biol.* (1998) 10:373-383); the p65 subunit of nuclear factor kappa B (Bitko & Barik, *J Virol.* (1998) 72:5610-5618 and Doyle & Hunt, *Neuroreport* (1997) 8:2937-2942); and EGR-I (early growth response gene product-1) (Yan *et al.*, *Proc. Nad. Acad. Sci. USA* (1998) 95:8298-8303; and Liu *et al.*, *Cancer Gene Ther.* (1998) 5:3-28).

Kinases, phosphatases, and other proteins that modify polypeptides involved in gene regulation are also useful as regulatory domains for zinc finger proteins. Such modifiers are often involved in switching on or off transcription mediated by, for example, hormones. Kinases involved in transcription regulation are reviewed in Davis, *Mol. Reprod. Dev.* (1995) 42:459-467, Jackson *et al.*, *Adv. Second Messenger Phosphoprotein Res.* (1993) 28:279-286, and Boulikas, *Crit. Rev. Eukaryot. Gene Expr.* (1995) 5:1-77, while phosphatases are reviewed in, for example, Schonthal & Semin, *Cancer Biol.* (1995) 6:239-248. Nuclear tyrosine kinases are described in Wang, *Trends Biochem. Sci.* (1994) 19:373-376.

Useful domains can also be obtained from the gene products of oncogenes (*e.g.*, myc, jun, fos, myb, max, mad, rel, ets, bcl, myb, mos family members) and their associated factors and modifiers. Oncogenes are described in, for example, Cooper, *Oncogenes*, 2nd ed., The Jones and Bartlett Series in Biology, Boston, MA, Jones and Bartlett Publishers, 1995. The ets transcription factors are reviewed in Waslylk *et al.*, *Eur. J Biochem.* (1993) 211:7-18. Myc oncogenes are reviewed in, for example, Ryan *et* 30

al., *Biochem. J.* (1996) 314:713-721. The Jun and fos transcription factors are described in, for example, The Fos and Jun Families of Transcription Factors, Angel & Herrlich, eds. (1994). The max oncogene is reviewed in Hurlin *et al.*, *Cold Spring Harb. Symp. Quant. Biol.* 59:109-116. The myb gene family is reviewed in Kanei-Ishii *et al.*, *Curr. Top. Microbiol. Immunol.* (1996) 211:89-98. The mos family is reviewed in Yew *et al.*, *Curr. Opin. Genet. Dev.* (1993) 3:19-25.

In still another embodiment, histone acetyltransferase is used as a transcriptional activator (see, e.g., Jin & Scotto, *Mol. Cell. Biol.* (1998) 18:4377-4384; Wolffle, *Science* (1996) 272:371-372; Taunton *et al.*, *Science* (1996) 272:408-411; and Hassig *et al.*, *Proc. Natl. Acad. Sci. USA* (1998) 95:3519-3524). In another embodiment, histone deacetylase is used as a transcriptional repressor (see, e.g., Jin & Scotto, *Mol. Cell. Biol.* (1998) 18:4377-4384; Syntichaki & Thireos, *J Biol. Chem.* (1998) 273:24414-24419; Sakaguchi *et al.*, *Genes Dev.* (1998) 12:2831-2841; and Martinez *et al.*, *J Biol. Chem.* (1998) 273:23781-23785).

In a preferred embodiment, the plant effectors listed in the following Table 1 can be used:

Table 1. Exemplary plant effectors.

Gene	Organism	Activator	Repressor	Region Cloned (aa)	GenBank Accession	Plasmid	Reference
APETALA (AP1)	Arabidopsis	yes		194-255	S27109	AP1 Effector	Plant Molecular Biology 40:419-429 (1999)
DNA binding with one finger (Dof1)	Maize	yes		135-238	X66076	Dof1 Effector	The Plant Cell 10:75-89 (1998)
Dof2	Maize		yes	112-221	S59852	mDof2 Effector	The Plant Cell 10:75-89 (1998)
Dof2	Arabidopsis			115-204	BAA33197	AtDof2 Effector	Blast Search
Viral Protein 16 (VP16)	Herpes Virus	yes				VP16 Effector	

C1	maize	yes				C1 Effector	
G-box binding factor (GBF-1)	Arabidopsis	yes		1-110	P42774	GBF1 Effector	EMBO 11(4):1275-1289 (1992)
Mybst1	Potato			173-358		Mybst1 Effector	EMBO 13(22):5383-5392 (1994)
PCP1	Potato			206-496	X82328	PCP1 Effector	Mol. Gen. Genet. 247: 759-763 (1995)
PWRKY1	Parsley	yes		1-187	U48831	Pwrky1 Effector	EMBO 18(17): 4689-4699 (1999)
PWRKY3	Parsley			1-237		Pwrky3 Effector	EMBO 18(17): 4689-4699 (1999)
AFT1 (14-3-3 protein)	Arabidopsis	yes		1-248	U02565	AFT1 Effector	FEBS Letters 443: 282-284 (1999)
ZAP1	Arabidopsis	yes		168-282	X92976	Zap1 Effector	Nucleic Acids Research 24(23): 4624-4631 (1996)

Target genes and target nucleotide sequences

Expression of any target genes in plant can be modulated by the present method.

For example, the expression of the APETALA3 (AP3) gene of Arabidopsis can be modulated. The APETALA3 (AP3) gene of Arabidopsis is a member of the ADS domain proteins that are required to specify the flower organ types and are involved in regulation of floral development. The success illustrated below in targeting this gene for endogenous gene regulation is important for several reasons. First, Arabidopsis is a well-studied model organism in which genetic studies are easily and rapidly performed and is considered a typical case. Second, AP3 has been well studied in its role during flower development to specify floral organ identity (see, Yanofsky, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* (1995) 46:167-188; and Irish, *Developmental Biology* (1999) 209:211-

220) and detailed analysis of the AP3 promoter and its transcription have already been published (Irish *et al.*, *The Plant Cell* (1995) 7:1635-1644) so that the approach is illustrative for other plants as well. Finally, both ap3 mutants and AP3 overexpressors have previously been shown to have visible flower phenotypic changes (Jack *et al.*, *Cell* (1992) 68:683-687; and Jack *et al.*, *Cell* (1994) 76:703-716), which makes success verifiable. Thus, the AP3 gene is an excellent target for demonstrating this technology as a tool for controlling gene expression in plants in general.

In another example, the expression of the maize MIPS gene can be modulated. The maize MIPS protein catalyzes the conversion of glucose-6-phosphate to *myoinositol* 1-phosphate which is an early step in the synthesis of phytic acid. Low phytic acid content in feed for many species of animals is considered to have a great advantage from environmental and nutritional standpoints since phytic acid metabolism in plant plays a role in the regulation of phosphate and mineral concentration. Controlling the expression of the MIPS gene would therefore provide a means to assure plants with low phytic acid content and act as a dominant trait. The present invention permits such control, as well as control of genetic expression generally in plants.

The target nucleotide sequence is any location within the target gene whose expression is to be regulated which provides a suitable location for controlling expression. The target nucleotide sequence may be within the coding region or upstream or downstream thereof. For activation, upstream from ATG translation start codon is preferred, most preferably upstream of TATTA box but not exceeding 1000 bp from the start of transcription. For repression, upstream from the ATG translation start codon is also preferred, but preferably downstream from TATTA box.

The targeted nucleotide sequence can also be a short portion of duplex nucleic acid, *e.g.*, RNA, DNA, PNA or any hybrids thereof, having from about 8 to about 40 base pairs and having a defined sequence for which there is some desirable purpose in determining its presence or absence within a larger polynucleotide. For example, it may be desirable to determine whether a particular promoter or control region is found within the genome of a particular organism. A labeled protein, *e.g.*, bound with a radioactive or fluorescent label, containing zinc fingers which binds to a polynucleotide having this

particular sequence can be used to determine whether the genetic material of the organism contains this particular sequence.

The target sequence may reside endogenously in the target gene, e.g., MIPS in maize and AP3 in Arabidopsis as shown in the Example Section below, or may be inserted into the gene, e.g., heterologous, as is illustrated below for luciferase in tobacco, for example, using techniques such as homologous recombination.

Where a gene contains a suitable target nucleotide sequence in a region which is appropriate for controlling expression, the regulatory factors employed in the methods of the invention can target the endogenous nucleotide sequence. However, if the target gene lacks an appropriate unique nucleotide sequence or contains such a sequence only in a position where binding to a regulatory factor would be ineffective in controlling expression, it may be necessary to provide a "heterologous" targeted nucleotide sequence. By "heterologous" targeted nucleotide sequence is meant either a sequence completely foreign to the gene to be targeted or a sequence which resides in the gene itself, but in a different position from that wherein it is inserted as a target. Thus, it is possible to completely control the nature and position of the targeted nucleotide sequence.

The target sequence may be any given sequence of interest for which a complementary zinc finger protein is designed. Target genes include both structural and regulatory genes. When the target gene is a regulatory gene, the expression of the gene that is regulated by the target regulatory gene can also be regulated by the zinc finger protein, albeit indirectly. Therefore, expression of single genes or gene families can be controlled by the present methods.

The target gene may, as is the case for the MIPS gene and AP3 gene, be endogenous to the plant cells or plant wherein expression is regulated or may be a transgene which has been inserted into the cells or plants in order to provide a production system for a desired protein or which has been added to the genetic compliment in order to modulate the metabolism of the plant or plant cells, for example as illustrated below for the C7 binding site inserted into tobacco genome.

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Selection of ZFP, effector and target nucleotide sequence

The assay system exemplified in Example 1 (see Example Section below) can be used in selecting desirable ZFP, effector or target nucleotide sequence. The assay system used in the Example 1 generally contains two constructs. One construct contains a reporter gene, *e.g.*, P-glucuronidase (GUS), chloramphenicol acetyl transferase (CAT), or green fluorescent protein (GFP), operably linked to a promoter having a ZFP target nucleotide sequence so that the expression of the reporter gene is amenable to regulation by the ZFP. The other construct contains ZFP gene, and optionally fused to an effector domain. Upon introduction into and expression within plant or plant cells, whether transiently or stably, the ZFP, and optionally the ZFP-effector fusion protein, will regulate the expression of the reporter gene, provided that the ZFP and the target nucleotide sequence have matching "code" sequence, *e.g.*, the pairs listed in Table 2. In such a system, if only one component of the system is changed and the rest of the components are kept constant with the known operative ones, the assay system can be used to screen or select for new ZFP, effector or target nucleotide sequence with desirable specificity or binding affinity.

For example, if a system is started with a ZFP, optionally with an effector and a target nucleotide sequence that are compatible and substitute the ZFP with a new amino acid sequence, it will be possible to determine whether the new amino acid sequence can function as a ZFP with the desired specificity and affinity to the particular target nucleotide sequence by comparing the assay readout with the known ZFP and the new amino acid sequence. New effector and target nucleotide sequence can be identified using similar procedure. If desired, the assay can be conducted quantitatively to identify ZFP, effector or target nucleotide sequence with particular specificity and binding affinity.

Since ZFPs can tolerate changes in the finger region, one can start with a preferred pairs of ZFP and target nucleotide sequence, *e.g.*, the pairs listed in Table 2, and mutagenize the finger region of the ZFP to identify those variants that can still function as a ZFP, but with different specificity to binding affinity. It is often necessary to select ZFP finger sequence by testing against actual DNA sequence, since the juxtaposition of triplets may affect optimal ZFP finger sequence. In addition, particular positions in the

finger region, e.g., -1, 3, 6, should be the focused in the mutagenesis analysis because these positions are thought to be critical for the binding between a ZFP and its target nucleotide sequence. Accordingly, ZFPs with different level of specificities and binding affinities can be obtained and these ZFPs can be used in fine-tuned control of a target gene expression in plant.

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C. Expression of zinc finger protein in plant and uses thereof

The zinc finger protein can be provided to the plant cells via any suitable methods known in the art. For example, the zinc finger protein can be exogenously added to the plant cells and the plant cells are maintained under conditions such that the zinc finger protein binds to the target nucleotide sequence and regulates the expression of the target gene in the plant cells. Alternatively, a nucleotide sequence encoding the zinc finger protein can be expressed in the plant cells and the plant cells are maintained under conditions such that the expressed zinc finger protein binds to the target nucleotide sequence and regulates the expression of the target gene in the plant cells.

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The zinc finger gene can be expressed in plant with any suitable plant expression vectors. Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, *Meth. in Enzymol.* (1987) 153:253-277. These vectors are plant integrating vectors in that upon transformation, the vectors integrate a portion of vector DNA into the genome of the host plant. Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, *Gene* (1987) 61:1-11 and Berger *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) 86:8402-8406. Another useful vector herein is plasmid pBI 101.2 that is available from Clontech Laboratories, Inc. (Palo Alto, CA).

In addition to regulatory domains, often the zinc finger protein can be expressed as a fusion protein such as maltose binding protein ("MBP"), glutathione S transferase (GST), hexahistidine, c-myc, and the FLAG epitope, for ease of purification, monitoring expression, or monitoring cellular and subcellular localization.

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The nucleic acid sequence encoding a ZFP can be modified to improve expression of the ZFP in plants by using codon preference. When the nucleic acid is prepared or

altered synthetically, advantage can be taken of known codon preferences of the intended plant host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific 5 codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (*Murray et al., Nucl. Acids Res.* (1989) 17: 477-498). Thus, the maize preferred codon for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants are listed in Table 4 of *Murray et al.*, supra.

In one embodiment, the transgene may include a reporter protein such as luciferase, which can be helpful in providing an assay system for determining the position 10 in a target gene in which the targeted nucleotide sequence should reside. Thus, the invention also includes an assay system for determination of a suitable region for targeting by regulatory factors, wherein said assay method comprises providing a chimeric gene comprising a nucleotide sequence encoding a reporter protein optionally fused to coding regions of said gene; said nucleotide sequence operably linked to control 15 sequences endogenous to said gene. The chimeric gene is then provided a targeted nucleotide sequence at various locations within the endogenous portions and the effect on production of the reporter protein of contact with the appropriate zinc finger containing regulatory protein is determined.

It may be desirable in some instances to modify plant cells or plants with families 20 of transgenes representing, for example, a metabolic pathway. In those instances, it may be desirable to design the constructs so that the family can be regulated as a whole, e.g., by designing the control regions of the members of the family with similar or identical targets for the zinc finger protein portion of the effector protein. Such sharing of target 25 sequences in gene families may occur naturally in endogenously produced metabolic sequences.

In most instances, it is desirable to provide the expression system for the effector 30 protein with control sequences that are tissue specific so that the desired gene regulation can occur selectively in the desired portion of the plant. For example, to repress MIPS expression, it is desirable to provide the effector protein with control sequences that are

selectively effective in seeds. With respect to the AP3 gene, effector proteins for regulation of expression would be designed for selective expression in flowering portions of the plant. However, in some instances, it may be desirable to have the genetic control expressible in all tissues in instances where an insect resistance gene is the target. In 5 such cases, it may be desirable to place the expression system for the effector protein under control of an inducible promoter so that inducer can be supplied to the plant only when the need arises, for example, activation of an insect resistance gene.

In another embodiment, ZFPs can be used to create functional "gene knockouts" and "gain of function" mutations in a host cell or plant by repression or activation of the 10 target gene expression. Repression or activation may be of a structural gene, *e.g.*, one encoding a protein having for example enzymatic activity, or of a regulatory gene, *e.g.*, one encoding a protein that in turn regulates expression of a structural gene. Expression of a negative regulatory protein can cause a functional gene knockout of one or more genes, under its control. Conversely, a zinc finger having a negative regulatory domain 15 can repress a positive regulatory protein to knockout or prevent expression of one or more genes under control of the positive regulatory protein.

The present invention further provides recombinant expression cassettes comprising a ZFP-encoding nucleic acid. A nucleic acid sequence coding for the desired polynucleotide of the present invention can be used to construct a recombinant expression cassette which can be introduced into the desired host cell. A recombinant expression cassette will typically comprise a polynucleotide encoding creation zinc finger proteins, *e.g.*, ZFPm1, ZFPm2, ZFPm3, ZFPm4 and ZFPAp3, operably linked to transcriptional initiation regulatory sequences which will direct the transcription of the polynucleotide in the intended host cell, such as tissues of a transformed plant.

For example, plant expression vectors may include (1) a cloned plant gene under 25 the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (*e.g.*, one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a 30 transcription termination site, and/or a polyadenylation signal.

A plant promoter fragment can be employed which will direct expression of a polynucleotide of the present invention in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the rice actin 1 promoter (U.S. Patent No. 5,641,876), the cauliflower mosaic virus (CaMV) 35S transcription initiation region (Odell *et al.*, *Nature* (1985) 313:810-812), the P- or 2'- promoter derived from T-DNA of Agrobacterium tumefaciens, the ubiquitin I promoter, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (U.S. Patent No. 5,683,439), the Nos promoter, the pEmu promoter, the rubisco promoter, the GRP 1 - 8 promoter, and other transcription initiation regions from various plant genes known to those of skilled artisans.

Alternatively, the plant promoter can direct expression of a polynucleotide of the present invention in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here as "inducible" promoters. Environmental conditions that may effect transcription by inducible promoters include pathogen attack, anaerobic conditions, or the presence of light. Examples of inducible promoters are the AdhI promoter which is inducible by hypoxia or cold stress, the Hsp70 promoter which is inducible by heat stress, and the PPDK promoter which is inducible by light. Examples of promoters under developmental control include promoters that initiate transcription only, or preferentially, in certain tissues, such as leaves, roots, fruit, seeds, or flowers. An exemplary promoter is the anther specific promoter 5126 (U.S. Patent Nos. 5,689,049 and 5,689,051). The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in certain locations.

Both heterologous and non-heterologous (*i.e.*, endogenous) promoters can be employed to direct expression of the nucleic acids of the present invention. These promoters can also be used, for example, in recombinant expression cassettes to drive expression of antisense nucleic acids to reduce, increase, or alter concentration and/or composition of the proteins of the present invention in a desired tissue. Thus, in some embodiments, the nucleic acid construct will comprise a promoter functional in a plant cell, such as in Zea mays, operably linked to a polynucleotide of the present invention.

In some embodiments, isolated nucleic acids which serve as promoter or enhancer elements can be introduced in the appropriate position (generally upstream) of a non-heterologous form of a polynucleotide of the present invention so as to up or down regulate expression of a polynucleotide of the present invention. For example,
5 endogenous promoters can be altered in vivo by mutation, deletion, and/or substitution (see, Kmiec, U.S. Patent No. 5,565,350; WO 93/22443), or isolated promoters can be introduced into a plant cell in the proper orientation and distance from a gene of the present invention so as to control the expression of the gene. Gene expression can be modulated under conditions suitable for plant growth so as to alter the total concentration
10 and/or alter the composition of the polypeptides of the present invention in plant cell.

The selection of the promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant, *i.e.*, the ZFP gene is only expressed in the desired tissue or at a certain time in plant development or growth. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and the selection will reflect the desired location of accumulation of the gene product. Alternatively, the selected promoter may drive expression of the gene under various inducing conditions. Additionally, the zinc finger encoding gene can be under control of and activated by a promoter responsive to the presence of a pathogen or to plant stress, *e.g.*, cold stress, salt stress, etc., so that the induced ZFP can modulate a gene that counteracts the pathogen, stress, etc. Promoters vary in their strength, *i.e.*, ability to promote transcription. Depending upon the host cell system utilized, any one of a number of suitable promoters can be used, including the gene's native promoter.
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A variety of promoters will be useful in the invention, particularly to control the expression of the ZFP and ZFP-effector fusions, the choice of which will depend in part upon the desired level of protein expression and desired tissue-specific, temporal specific, or environmental cue-specific control, if any in a plant cell. Constitutive and tissue specific promoters are of particular interest. Such constitutive promoters include, for example, the core promoter of the Rsyn7, the core CaMV 35S promoter (Odell *et al.*,
30 *Nature* (1985) 313:810-812), CaMV 19S (Gmunder and Kohli, *Mol Gen Genet* 1989

Dec; 220(1):95-101), rice actin (Wang et al., *Mol. Cell. Biol.*, 12, 3399-3406 (1992); U.S. Patent No. 5,641,876; and McElroy *et al.*, *Plant Cell* (1985) 2:163-171); ubiquitin (Christensen *et al.*, *Plant Mol. Biol.* (1989) 12:619-632 and Christensen *et al.*, *Plant Mol. Biol.* (1992) 18:675-689), pEMU (Last *et al.*, *Theor. Appl. Genet.* (1991) 81:581-588),
5 MAS (Veltenet *et al.*, *EMBO J.* (1984) 3:2723-2730), nos (Ebert *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84, 5745-5749 (1987)), Adh (Dennis and Berg, *Nucleic Acids Res.*, 13:7945-7957 (1985)), and constitutive promoters described in, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

10 Ubiquitin is a gene product known to accumulate in many cell types and its promoter has been cloned from several species for use in transgenic plants (e.g. sunflower - Binet *et al.*, *Plant Science* (1991) 79:87-94; maize - Christensen *et al.*, *Plant Molec. Biol.* (1989) 12: 619-632; and *Arabidopsis* - Norris *et al.*, *Plant Mol. Biol.* (1993) 21:895-906). The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence and vectors constructed for monocot transformation are disclosed in the patent publication EP 0 342 926 (to Lubrizol) which is herein incorporated by reference. Taylor *et al.* (*Plant Cell Rep.* (1993) 12:491-495) describe a vector (pAHC25) that comprises the maize ubiquitin promoter and first intron and its high activity in cell suspensions of numerous monocotyledons when introduced via microprojectile bombardment. The *Arabidopsis* ubiquitin promoter is also ideal for use with the nucleotide sequences of the present invention. The ubiquitin promoter is suitable for gene expression in transgenic plants, both monocotyledons and dicotyledons. Suitable vectors are derivatives of pAHC25 or any of the transformation vectors described in this application, modified by the introduction of the appropriate ubiquitin promoter and/or intron sequences.
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Construction of the plasmid pCGN1761 is described in the published patent application EP 0 392 225 (Example 23), which is hereby incorporated by reference. pCGN1761 contains the "double" CaMV 35S promoter and the *tml* transcriptional terminator with a unique *EcoRI* site between the promoter and the terminator and has a pUC-type backbone. A derivative of pCGN1761 is constructed which has a modified polylinker which includes *NotI* and *XhoI* sites in addition to the existing *EcoRI* site. This
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derivative is designated pCGN1761ENX. pCGN1761ENX is useful for the cloning of cDNA sequences or coding sequences (including microbial ORF sequences) within its polylinker for the purpose of their expression under the control of the 35S promoter in transgenic plants. The entire 35S promoter-coding sequence-*tml* terminator cassette of such a construction can be excised by *HindIII*, *SphI*, *SalI*, and *XbaI* sites 5' to the promoter and *XbaI*, *BamHI* and *BglII* sites 3' to the terminator for transfer to transformation vectors such as those described below. Furthermore, the double 35S promoter fragment can be removed by 5' excision with *HindIII*, *SphI*, *SalI*, *XbaI*, or *PstI*, and 3' excision with any of the polylinker restriction sites (*EcoRI*, *NotI* or *XhoI*) for replacement with another promoter. If desired, modifications around the cloning sites can be made by the introduction of sequences that may enhance translation. This is particularly useful when overexpression is desired. For example, pCGN1761ENX may be modified by optimization of the translational initiation site as described in Example 37 of U.S. Patent No. 5,639,949, incorporated herein by reference.

Several isoforms of actin are known to be expressed in most cell types and consequently the actin promoter is a good choice for a constitutive promoter. In particular, the promoter from the rice *ActI* gene has been cloned and characterized (McElroy *et al.*, *Plant Cell* (1990) 2:163-171). A 1.3 kb fragment of the promoter was found to contain all the regulatory elements required for expression in rice protoplasts. Furthermore, numerous expression vectors based on the *ActI* promoter have been constructed specifically for use in monocotyledons (McElroy *et al.*, *Mol. Gen. Genet.* (1991) 231:150-160). These incorporate the *ActI*-intron 1, *AdhI* 5' flanking sequence and *AdhI*-intron 1 (from the maize alcohol dehydrogenase gene) and sequence from the CaMV 35S promoter. Vectors showing highest expression were fusions of 35S and *ActI* intron or the *ActI* 5' flanking sequence and the *ActI* intron. Optimization of sequences around the initiating ATG (of the GUS reporter gene) also enhanced expression. The promoter expression cassettes described by McElroy *et al.* (*Mol. Gen. Genet.* (1991) 231:150-160) can be easily modified for gene expression and are particularly suitable for use in monocotyledonous hosts. For example, promoter-containing fragments are removed from the McElroy constructions and used to replace the double 35S promoter in pCGN1761ENX, which is then available for the insertion of specific gene sequences.

The fusion genes thus constructed can then be transferred to appropriate transformation vectors. In a separate report, the rice *ActI* promoter with its first intron has also been found to direct high expression in cultured barley cells (Chibbar *et al.*, *Plant Cell Rep.* (1993) 12:506-509).

5 Tissue-specific promoters can be utilized to target enhanced expression within a particular plant tissue (*See generally*, copending U.S. Provisional Patent Application entitled "Promoters for Regulation of Plant Gene Expression, Attorney Docket No. 1360.002PRV (Schwegman, Lundberg, Woessner & Kluth), filed June 23, 2000, the content of which is herein incorporated by reference in its entirety. Examples of tissue

10 specific promoters which have been described include the lectin (Vodkin, *Prog. Clin. Biol. Res.*, 138:87-98 (1983); and Lindstrom *et al.*, *Dev. Genet.*, 11:160-167 (1990)), corn alcohol dehydrogenase 1 (Dennis *et al.*, *Nucleic Acids Res.*, 12:3983-4000 (1984)), corn light harvesting complex (Becker, *Plant Mol Biol.*, 20(1): 49-60 (1992); and Bansal *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 89:3654-3658 (1992)), corn heat shock protein (Odell *et al.*, *Nature* (1985) 313:810-812; and Marrs *et al.*, *Dev. Genet.*, 14(1):27-41 (1993)), small subunit RuBP carboxylase (Waksman *et al.*, *Nucleic Acids Res.*, 15(17):7181 (1987); and Berry-Lowe *et al.*, *J. Mol. Appl. Genet.*, 1(6):483-498 (1982)), Ti plasmid mannopine synthase (Ni *et al.*, *Plant Mol. Biol.*, 30(1):77-96 (1996)), Ti plasmid nopaline synthase (Bevan, *Nucleic Acids Res.*, 11(2):369-385 (1983)), petunia chalcone isomerase (Van Tunen *et al.*, *EMBO J.*, 7:1257-1263 (1988)), bean glycine rich protein 1 (Keller *et al.*, *Genes Dev.*, 3:1639-1646 (1989)), truncated CaMV 35s (Odell *et al.*, *Nature* (1985) 313:810-812), potato patatin (Wenzler *et al.*, *Plant Mol. Biol.*, 13:347-354 (1989)), root cell (Yamamoto *et al.*, *Nucleic Acids Res.*, 18:7449 (1990)), maize zein (Reina *et al.*, *Nucleic Acids Res.*, 18:6425 (1990); Kriz *et al.*, *Mol. Gen. Genet.*, 207:90-98 1987;

15 20 Wandelt and Feix, *Nucleic Acids Res.*, 17:2354 (1989); Langridge and Feix, *Cell*, 34:1015-1022 (1983); and Reina *et al.*, *Nucleic Acids Res.*, 18:7449 (1990)), globulin-1 (Belanger *et al.*, *Genetics*, 129:863-872 (1991)), α -tubulin, cab (Sullivan *et al.*, *Mol. Gen. Genet.*, 215:431-440 (1989)), PEPCase (Cushman *et al.*, *Plant Cell*, 1(7):715-25 (1989)), R gene complex-associated promoters (Chandler *et al.*, *Plant Cell*, 1:1175-1183 (1989)), and chalcone synthase promoters (Franken *et al.*, *EMBO J.*, 10:2605-2612, 1991)).

Tissue-specific promoters disclosed in the following references may also be used:

Yamamoto *et al.*, *Plant J* (1997) 12(2):255-265; Kawamata *et al.*, *Plant Cell Physiol.* (1997) 38(7):792-803; Hansen *et al.*, *Mol. Gen Genet.* (1997) 254(3):337); Russell *et al.*, *Transgenic Res.* (1997) 6(2):157-168; Rinehart *et al.*, *Plant Physiol.* (1996) 112(3):1331; Van Camp *et al.*, *Plant Physiol.* (1996) 112(2):525-535; Canevascini *et al.*, *Plant Physiol.* (1996) 112(2):513-524; Yamamoto *et al.*, *Plant Cell Physiol.* (1994) 35(5):773-778; Lam, *Results Probl. Cell Differ.* (1994) 20:181-196; Orozco *et al.*, *Plant Mol. Biol.* (1993) 23(6):1129-1138; Matsuoka *et al.*, *Proc Natl. Acad. Sci. USA* (1993) 90(20):9586-9590; and Guevara-Garcia *et al.*, *Plant J.* (1993) 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

A maize gene encoding phosphoenol carboxylase (PEPC) has been described by Hudspeth & Grula (*Plant Molec Biol* 12: 579-589 (1989)). Using standard molecular biological techniques the promoter for this gene can be used to drive the expression of any gene in a leaf-specific manner in transgenic plants. Other leaf-specific promoters are known in the art, and include those described in, for example, Yamamoto *et al.*, *Plant J.* (1997) 12(2):255-265; Kwon *et al.*, *Plant Physiol.* (1994) 105:357-367; Yamamoto *et al.*, *Plant Cell Physiol.* (1994) 35(5):773-778; Gotor *et al.*, *Plant J.* (1993) 3:509-518; Orozco *et al.*, *Plant Mol. Biol.* (1993) 23(6):1129-1138; and Matsuoka *et al.*, *Proc. Natl. Acad. Sci. USA* (1993) 90(20):9586-9590.

Another pattern of gene expression is root expression. A suitable root promoter is the promoter of the maize metallothionein-like (MTL) gene described by de Framond (*FEBS* (1991) 290:103-106) and also in U.S. Patent No. 5,466,785, incorporated herein by reference. This "MTL" promoter is transferred to a suitable vector such as pCGN1761ENX for the insertion of a selected gene and subsequent transfer of the entire promoter-gene-terminator cassette to a transformation vector of interest. Other examples of root-specific promoters which have been described include the RB7 promoter from *Nicotiana tabacum* (U.S. Patent Nos. 5,459,252 and 5,750,386).

WO 93/07278 describes the isolation of the maize calcium-dependent protein kinase (CDPK) gene which is expressed in pollen cells. The gene sequence and promoter extend up to 1400 bp from the start of transcription. Using standard molecular biological techniques, this promoter or parts thereof, can be transferred to a vector such as

pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a nucleic acid sequence of the invention in a pollen-specific manner.

Inducible promoters and other types of regulated promoters can also be used. For example, PR-1 promoters, ethanol-inducible promoters, glucocorticoid-inducible 5 promoters, wound-inducible promoters, promoters for pith-preferred expression and promoters for receptor mediated transactivation in the presence of a chemical ligand can be used.

The double 35S promoter in pCGN1761ENX may be replaced with any other promoter of choice that will result in suitably high expression levels. By way of 10 example, one of the chemically regulatable promoters described in U.S. Patent No. 5,614,395, such as the tobacco PR-1a promoter, may replace the double 35S promoter.

Alternately, the *Arabidopsis* PR-1 promoter described in Lebel *et al.*, *Plant J.* (1998) 15 16:223-233 may be used. The promoter of choice is preferably excised from its source by restriction enzymes, but can alternatively be PCR-amplified using primers that carry appropriate terminal restriction sites. Should PCR-amplification be undertaken, then the promoter should be re-sequenced to check for amplification errors after the cloning of the amplified promoter in the target vector. The chemically/pathogen regulatable tobacco PR-1a promoter is cleaved from plasmid pCIB1004 (for construction, see example 21 of EP 0 332 104, which is hereby incorporated by reference) and transferred to plasmid 20 pCGN1761ENX (U.S. Patent No. 5,639,949). pCIB1004 is cleaved with *NcoI* and the resultant 3' overhang of the linearized fragment is rendered blunt by treatment with T4 DNA polymerase. The fragment is then cleaved with *HindIII* and the resultant PR-1a promoter-containing fragment is gel purified and cloned into pCGN1761ENX from 25 which the double 35S promoter has been removed. This is done by cleavage with *XhoI* and blunting with T4 polymerase, followed by cleavage with *HindIII* and isolation of the larger vector-terminator containing fragment into which the pCIB1004 promoter fragment is cloned. This generates a pCGN1761ENX derivative with the PR-1a promoter and the *tml* terminator and an intervening polylinker with unique *EcoRI* and 30 *NotI* sites. The selected coding sequence can be inserted into this vector, and the fusion products (*i.e.* promoter-gene-terminator) can subsequently be transferred to any selected transformation vector, including those described *infra*. Various chemical regulators may

be employed to induce expression of the selected coding sequence in the plants transformed according to the present invention, including the benzothiadiazole, isonicotinic acid, and salicylic acid compounds disclosed in U.S. Patent Nos. 5,523,311 and 5,614,395.

5 A promoter inducible by certain alcohols or ketones, such as ethanol, may also be used to confer inducible expression of a coding sequence of the present invention. Such a promoter is for example the *alcA* gene promoter from *Aspergillus nidulans* (Caddick *et al.*, (1998) *Nat. Biotechnol* 16:177-180). In *A. nidulans*, the *alcA* gene encodes alcohol dehydrogenase I, the expression of which is regulated by the AlcR transcription factors in 10 presence of the chemical inducer. For the purposes of the present invention, the CAT coding sequences in plasmid palcA:CAT comprising a *alcA* gene promoter sequence fused to a minimal 35S promoter (Caddick *et al.*, *Nat. Biotechnol* (1998) 16:177-180) are replaced by a coding sequence of the present invention to form an expression cassette having the coding sequence under the control of the *alcA* gene promoter. This is carried 15 out using methods well known in the art.

15 Induction of expression of a nucleic acid sequence of the present invention using systems based on steroid hormones is also contemplated. For example, a glucocorticoid-mediated induction system is used (Aoyama and Chua, *The Plant Journal* (1997) 11:605-612) and gene expression is induced by application of a glucocorticoid, for example a synthetic glucocorticoid, preferably dexamethasone, preferably at a concentration ranging 20 from 0.1mM to 1mM, more preferably from 10mM to 100mM. For the purposes of the present invention, the luciferase gene sequences are replaced by a nucleic acid sequence of the invention to form an expression cassette having a nucleic acid sequence of the invention under the control of six copies of the GAL4 upstream activating sequences fused to the 35S minimal promoter. This is carried out using methods well known in the 25 art. The trans-acting factor comprises the GAL4 DNA-binding domain (Keegan *et al.*, *Science* (1986) 231:699-704) fused to the transactivating domain of the herpes viral protein VP16 (Triebenbarg *et al.*, *Genes Devel.* (1988) 2:718-729) fused to the hormone-binding domain of the rat glucocorticoid receptor (Picard *et al.*, (1988) *Cell* 54:1073-1080). The expression of the fusion protein is controlled by any promoter suitable for 30 expression in plants known in the art or described here. This expression cassette is also

comprised in the plant comprising a nucleic acid sequence of the invention fused to the 6xGAL4/minimal promoter. Thus, tissue- or organ-specificity of the fusion protein is achieved leading to inducible tissue- or organ-specificity of the insecticidal toxin.

Wound-inducible promoters may also be suitable for gene expression. Numerous such promoters have been described (e.g. Xu *et al.*, *Plant Molec. Biol.* (1993) 22:573-588; Logemann *et al.*, *Plant Cell* (1989) 1:151-158; Rohrmeier & Lehle, *Plant Molec. Biol.* (1993) 22:783-792; Firek *et al.*, *Plant Molec. Biol.* (1993) 22:129-142; and Warner *et al.*, *Plant J.* (1993) 3:191-201) and all are suitable for use with the instant invention. Logemann *et al.* describe the 5' upstream sequences of the dicotyledonous potato *wunI* gene. Xu *et al.* show that a wound-inducible promoter from the dicotyledon potato (*pin2*) is active in the monocotyledon rice. Further, Rohrmeier & Lehle describe the cloning of the maize *WipI* cDNA which is wound induced and which can be used to isolate the cognate promoter using standard techniques. Similar, Firek *et al.* and Warner *et al.* have described a wound-induced gene from the monocotyledon *Asparagus officinalis*, which is expressed at local wound and pathogen invasion sites. Using cloning techniques well known in the art, these promoters can be transferred to suitable vectors, fused to the genes pertaining to this invention, and used to express these genes at the sites of plant wounding.

WO 93/07278, which is herein incorporated by reference, describes the isolation of the maize *trpA* gene, which is preferentially expressed in pith cells. The gene sequence and promoter extending up to -1726 bp from the start of transcription are presented. Using standard molecular biological techniques, this promoter, or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a foreign gene in a pith-preferred manner. In fact, fragments containing the pith-preferred promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants.

U.S. Patent No. 5,880,333 describes a system whereby class II hormone receptors such as Ecdysone Receptor (EcR) and Ultraspiracle (USP), which function together as a heterodimer, regulate the expression of a target polypeptide in a plant cell in the presence of an appropriate chemical ligand, e.g., tebufenozide.

Particularly preferred are the inducible PR1 promoter, maize ubiquitin promoter, and rice actin promoter.

Any combination of constitutive or inducible and non-tissue specific or tissue specific may be used to control ZFP expression.

5 The desired control may be temporal, developmental or environmentally controlled using the appropriate promoter. Environmentally controlled promoters are those that respond to assault by pathogen, pathogen toxin, or other external compound (e.g., intentionally applied small molecule inducer). An example of a temporal or developmental promoter is a fruit ripening-dependent promoter.

10 Thus, the present invention provides compositions, and methods for making, heterologous promoters and/or enhancers operably linked to a ZFP and ZFP-effector fusion encoding polynucleotide of the present invention.

15 By selection of different strength of promoter to express ZFP-effector fusion protein, one can control the effect of ZFP in gene expression regulation in many levels. Generally, The stronger the promoter is, the stronger the effect of ZFP-effector protein is. However, moderate level of gene expression activation or repression is desirable since over-expression of certain foreign protein or over-activation of certain endogenous gene in certain cell type may cause toxicity effect. As in the example of AP3 gene activation, the Ubiquitin promoter (strong promoter) was originally used to express ZFP Ap3-VP64 activation fusion protein. We have switched to a weaker promoter AP3 endogenous promoter to express the same constructs to achieve even clear phenotype changes.

20 Methods for identifying promoters with a particular expression pattern, in terms of, e.g., tissue type, cell type, stage of development, and/or environmental conditions, are well known in the art. See, e.g., The Maize Handbook, Chapters 114-115, Freeling and Walbot, Eds., Springer, New York (1994); Corn and Corn Improvement, Pediton, Chapter 6, Sprague and Dudley, Eds., American Society of Agronomy, Madison, Wisconsin (1988).

25 A typical step in promoter isolation is identification of gene products that are expressed with some degree of specificity in the target tissue. Amongst the range of methodologies are: differential hybridization to cDNA libraries; subtractive hybridization; differential display; differential 2-D protein gel electrophoresis; DNA

probe arrays; and isolation of proteins known to be expressed with some specificity in the target tissue. Such methods are well known to those of skill in the art. Commercially available products for identifying promoters are known in the art such as Clontech's (Palo Alto, CA) Universal GenomeWalker Kit.

5 For the protein-based methods, it is helpful to obtain the amino acid sequence for at least a portion of the identified protein, and then to use the protein sequence as the basis for preparing a nucleic acid that can be used as a probe to identify either genomic DNA directly, or preferably, to identify a cDNA clone from a library prepared from the target tissue. Once such a cDNA clone has been identified, that sequence can be used to identify the sequence at the 5' end of the transcript of the indicated gene. For differential hybridization, subtractive hybridization and differential display, the nucleic acid sequence identified as enriched in the target tissue is used to identify the sequence at the 5' end of the transcript of the indicated gene. Once such sequences are identified, starting 10 either from protein sequences or nucleic acid sequences, any of these sequences identified as being from the gene transcript can be used to screen a genomic library prepared from the target organism. Methods for identifying and confirming the transcriptional start site are well known in the art.

15 In the process of isolating promoters expressed under particular environmental conditions or stresses, or in specific tissues, or at particular developmental stages, a number of genes are identified that are expressed under the desired circumstances, in the desired tissue, or at the desired stage. Further analysis will reveal expression of each 20 particular gene in one or more other tissues of the plant. One can identify a promoter with activity in the desired tissue or condition but that do not have activity in any other common tissue.

25 To identify the promoter sequence, the 5' portions of the clones can be analyzed for sequences characteristic of promoter sequences. For instance, promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually an AT-rich stretch of 5-10 bp located approximately 20 to 40 base pairs upstream of the transcription start site. Identification of the TATA box is well known in the art. For 30 example, one way to predict the location of this element is to identify the transcription start site using standard RNA-mapping techniques such as primer extension, S I analysis,

and/or RNase protection. To confirm the presence of the AT-rich sequence, a structure-function analysis can be performed involving mutagenesis of the putative region and quantification of the mutation's effect on expression of a linked downstream reporter gene. See, e.g., The Maize Handbook, Chapter 114, Freeling and Walbot, Eds., Springer, New York (1994).

5 In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element (*i.e.*, the CAAT box) with a series of adenines surrounding the trinucleotide G (or T) N G (Messing *et al.*, in Genetic Engineering in Plants, Kosage, Meredith and Hollaender, Eds., pp. 221-227 1983). In maize, there is no well conserved
10 CAAT box but there are several short, conserved protein- binding motifs upstream of the TATA box. These include motifs for the trans-acting transcription factors involved in light regulation, anaerobic induction, hormonal regulation, or anthocyanin biosynthesis, as appropriate for each gene.

15 Once promoter and/or gene sequences are known, a region of suitable size is selected from the genomic DNA that is 5' to the transcriptional start, or the translational start site, and such sequences are then linked to a coding sequence. If the transcriptional start site is used as the point of fusion, any of a number of possible 5' untranslated regions can be used in between the transcriptional start site and the partial coding sequence. If the translational start site at the 3' end of the specific promoter is used, then it is linked
20 directly to the methionine start codon of a coding sequence.

25 If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene.

30 An intron sequence can be added to the 5' untranslated region or the coding sequence of the partial coding sequence to increase the amount of the mature message that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1,000-fold (Buchman and Berg,

Mol. Cell Biol. (1988) 8:4395-4405; and Callis *et al.*, *Genes Dev.* (1987) 1:1183-1200). Such intron enhancement of gene expression is typically greatest when placed near the 5' end of the transcription unit. Use of maize introns Adhl-S intron 1, 2, and 6, the Bronze-I intron are known in the art (See generally, The Maize Handbook, Chapter 116, Freeling and Walbot, Eds, Springer, New York (1994))

Plant transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.*, monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.*, *Biotechniques* (1986) 4:320-334), electroporation (Riggs *et al.*, *Proc. Natl. Acad Sci. USA* (1986) 83:5602-5606), Agrobacterium-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055), direct gene transfer (Paszkowski *et al.*, *EMBO J.* (1984) 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995). "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.*, *Biotechnology* (1988) 6:923-926). Also see Weissinger et al., *Ann. Rev. Genet.* (1988) 22:421-477; Sanford *et al.*, *Particulate Science and Technology* (1987) 5:27-37 (onion); Christou *et al.*, *Plant Physiol.* (1988) 87:671-674 (soybean); McCabe *et al.*, *BioTechnology* (1988) 6:923-926 (soybean); Finer and McMullen, *In Vitro Cell Dev. Biol.* (1991) 27P: 175-182 (soybean); Singh *et al.*, *Theor. Appl. Genet.* (1998) 96:319-324 (soybean); Datta *et al.*, *Biotechnology* (1990) 8:736-740 (rice); Klein *et al.*, *Proc. Natl. Acad Sci. USA* (1988) 85:4305-4309 (maize); Klein *et al.*, *Biotechnology* (1988) 6:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Busing *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al.* (1995). "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.*, *Plant Physiol.* (1988) 91:440-444 (maize); Fromm *et al.*, *Biotechnology* (1990) 8:833-839 (maize); Hooykaas-Van Slogteren *et al.*, *Nature (London)* (1984) 311:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.*, *Proc. Natl. Acad Sci. USA* (1987) 84:5345-5349 (Liliaceae). In The

Experimental Manipulation of Ovule Tissues, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaepller *et al.*, *Plant Cell Reports* (1990) 9:415- 418 and Kaepller *et al.*, *Theor. Appl. Genet.* (1992) 84:560-566 (whisker- mediated transformation); D'Halluin *et al.*, *Plant Cell* (1992) 4:1495-1505 (electroporation); Li *et al.*, *Plant Cell Reports* (1993) 12:250-255 and Christou and Ford, *Annals qf Botany* (1995) 75:407-413 (rice); Osjoda *et al.*, *Nature Biotechnology* (1996) 14:745-750 (maize via Agrobacterium tumefaciens); all of which are herein incorporated by reference.

The zinc finger protein with optional effector domain can be targeted to a specific organelle within the plant cell. Targeting can be achieved with providing the ZFP an appropriate targeting peptide sequence, such as a secretory signal peptide (for secretion or cell wall or membrane targeting, a plastid transit peptide, a chloroplast transit peptide, a mitochondrial target peptide, a vacuole targeting peptide, or a nuclear targeting peptide, and the like (Reiss *et al.*, *Mol. Gen. Genet.* (1987) 209(1):116-121; Settles and Martienssen, *Trends Cell Biol* (1998) 12:494-501; Scott *et al.*, *J Biol Chem* (2000) 10:1074; and Luque and Correas, *J Cell Sci* (2000) 113:2485-2495).

For examples, certain plastid organelle targeting sequences are disclosed in WO 00/12732 (see also de Castro Silva Filho *et al.*, *Plant Mol. Biol.* (1996) 30:769-1780; Schnell, D.J. *et al.*, *J Biol. Chem.* (1991) 266(5):3335-3342; and Lamppa *et al.*, *J Biol. Chem.* (1988) 263:14996-14999). Plastids are a class of plant organelles derived from proplastids and include chloroplasts, leucoplasts, aravloplasts, and chromoplasts. The plastids are major sites of biosynthesis in plants. In addition to photosynthesis in the chloroplast, plastids are also sites of lipid biosynthesis, nitrate reduction to ammonium, and starch storage. And while plastids contain their own circular genome, most of the proteins localized to the plastids are encoded by the nuclear genome and are imported into the organelle from the cytoplasm.

As described in the Example Section below, a nuclear localization peptide (see Figure 3) was used to target the ZFP-effector fusions to the nucleus.

The modified plant may be grown into plants in accordance with conventional ways (See, e.g., McCormick *et al.*, *Plant Cell. Reports* (1986) 81-84). These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or

more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

The method of the invention is particularly appealing to the plant breeder because it has the effect of providing a dominant trait, which minimizes the level of crossbreeding necessary to develop a phenotypically desirable species which is also commercially valuable. Typically, modification of the plant genome by conventional methods creates heterozygotes where the modified gene is phenotypically recessive. Crossbreeding is required to obtain homozygous forms where the recessive characteristic is found in the phenotype. This crossbreeding is laborious and time consuming. The need for such crossbreeding is eliminated in the case of the present invention which provides an immediate phenotypic effect.

When the target gene encodes a target protein, the present method can be used to modulate the expression of said encoded target protein. Expression of any target protein can be modulated by the present method in plant cells. The protein whose expression being modulated can be endogenous or exogenous to the plant cell. The modulation can be activation or inhibition.

In a specific embodiment, the protein whose expression being modulated is an antibody. In another specific embodiment, the protein whose expression being modulated participates in a metabolic pathway or controls a metabolic pathway, e.g., an anabolic or a catabolic pathway. The present method can be used for modulating metabolic pathways of any desirable molecules such as vitamins, taste molecules, e.g., bad taste molecules, anti-oxidants, sugars and flavanoids. The metabolic pathway being modulated can be endogenous or exogenous to the plant cell. In still another specific embodiment, target gene encodes a structural protein, e.g., an enzyme or a co-factor in a metabolic pathway, or a regulatory protein. In yet another specific embodiment, The metabolic pathway being modulated enhances an input or output trait in a plant or seed.

Enzyme activity means herein the ability of an enzyme to catalyze the conversion of a substrate into a product. A substrate for the enzyme comprises the natural substrate of the enzyme but also comprises analogues of the natural substrate, which can also be converted, by the enzyme into a product or into an analogue of a product. The activity of

the enzyme is measured for example by determining the amount of product in the reaction after a certain period of time, or by determining the amount of substrate remaining in the reaction mixture after a certain period of time. The activity of the enzyme is also measured by determining the amount of an unused co-factor of the reaction remaining in the reaction mixture after a certain period of time or by determining the amount of used co-factor in the reaction mixture after a certain period of time. The activity of the enzyme is also measured by determining the amount of a donor of free energy or energy-rich molecule (e.g., ATP, phosphoenolpyruvate, acetyl phosphate or phosphocreatine) remaining in the reaction mixture after a certain period of time or by determining the amount of a used donor of free energy or energy-rich molecule (e.g., ADP, pyruvate, acetate or creatine) in the reaction mixture after a certain period of time.

Co-factor is a natural reactant, such as an organic molecule or a metal ion, required in an enzyme-catalyzed reaction. A co-factor can be regenerated and reused. Exemplary co-factors include NAD(P), riboflavin (including FAD and FMN), folate, molybdopterin, thiamin, biotin, lipoic acid, pantothenic acid and coenzyme A, S-adenosylmethionine, pyridoxal phosphate, ubiquitinone, menaquinone.

D. Genetically modified plant cells, tissues and seeds

In yet another aspect, the present invention is directed to a genetically modified plant cell, which cell comprises an expression system for a zinc finger protein, said zinc finger protein is capable of binding, and preferably specifically binding, to a target nucleotide sequence, or a complementary strand thereof, within a target gene whose expression is to be modulated by said zinc finger protein.

The genetically modified plant cell can comprise any desirable target nucleotide sequence and target gene. Within the genetically modified plant cell, the target nucleotide sequence can be endogenous or exogenous to the targeted gene. The target gene can be endogenous or exogenous to the plant cells. The genetically modified plant cell can exist in culture or can be contained in an intact plant.

Within the genetically modified plant cell, the zinc finger protein can be used to control the expression of any target genes. In a specific embodiment, the zinc finger protein controls its own expression by binding to a target sequence within the zinc finger

protein gene. In another specific embodiment, the zinc finger protein controls its own expression by binding to a first target sequence within the zinc finger protein gene and controls the expression of the target gene by binding to a second target sequence within the target gene. Preferably, the first target sequence within the zinc finger protein gene is different from the second target sequence within the target gene. The expression of the zinc finger protein gene can be further controlled by second promoter, e.g., an inducible promoter. The zinc finger protein contained in the genetically modified plant cell can contain any number of zinc finger sequence. Preferably, the zinc finger protein comprises at least two zinc finger sequences, e.g., from about 2 to about 6 zinc finger sequences or from about 3 to about 6 zinc finger sequences.

Any plant cell can be genetically modified to comprise an expression system for a zinc finger protein, so that the expression of a target gene is modulated by said zinc finger protein. Exemplary plants include tobacco, tomato, potato, banana, soybean, pepper, wheat, rye, rice, spinach, carrot, maize and corn.

A method to modulate expression in a plant cell is provided, which method comprises culturing the genetically modified plant cell(s). Preferably, such genetically modified plant cell(s) is cultured in planta.

In a specific embodiment, a transgenic plant cell is provided, which plant cell is transformed with a nucleic acid comprising a functional geminiviral replicase gene operably linked to a fruit ripening-dependent promoter. In another specific embodiment, a genetically modified plant cell is provided, which cell comprises a heterologous zinc finger protein that specifically binds to a target nucleotide sequence in said plant cell wherein said heterologous zinc finger protein is constitutively or inducibly expressed.

A genetically modified plant tissue is also provided herein, which tissue comprises the above-described genetically modified plant cell(s).

A genetically modified plant seed, e.g., a tobacco, tomato, potato, banana, soybean, pepper, wheat, rye, rice, spinach, carrot, maize and corn seed, is further provide herein, which seed comprises the above-described genetically modified plant cell(s).

A transgenic plant seed is further provide herein, which seed is transformed with a nucleic acid comprising a functional geminiviral replicase gene operably linked to a fruit ripening-dependent promoter.

In a specific embodiment, the genetically modified plant cell, tissue, seed and whole plant does not contain a zinc finger protein that is disclosed in U.S. Patent No. 6,140,466 or WO 98/54311, e.g., a zinc finger-nucleotide binding polypeptide variant comprising at least three zinc finger modules that bind to a target cellular nucleotide sequence and modulate the transcriptional function of the cellular nucleotide sequence, wherein the amino acid sequence of each zinc finger module that binds a target cellular nucleotide comprises two cysteines and two histidines whereby both cysteines are amino proximal to both histidines and wherein each of three modules of said variant has at least one amino acid sequence modification.

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E. Nucleic acids encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 and ZFPAp3

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In yet another aspect, the present invention is directed to an isolated nucleic acid fragment, comprising a sequence of nucleotides encoding ZFPm1 (SEQ ID NO:14), ZFPm2 (SEQ ID NO:15), ZFPm3 (SEQ ID NO:16), ZFPm4 (SEQ ID NO:17) or ZFPAp3 (SEQ ID NO:18). The isolated nucleic acid fragment can be DNA or RNA. An isolated nucleic acid fragment, which is hybridizable to the nucleic acid fragment encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 under low, medium and high stringency condition is also provided. Preferably, the isolated nucleic acid fragment hybridizes to the nucleic acid fragment encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 under high stringency condition. Plasmids comprising the nucleic acid fragment encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 and cells comprising such plasmids are further provided. Any suitable cells can be used and preferably, bacterial cells, yeast cells, fungal cells, plant cells, insect cells and animal cells are used.

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The nucleic acid fragment encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 can be prepared by any methods known in the art, e.g., recombinant production, chemical synthesis or a combination thereof (See generally, *Current Protocols in Molecular Biology* (1998) § 20, John Wiley & Sons, Inc; Knorre, *Design and Targeted Reactions of Oligonucleotide Derivative*, CRC Press, 1994; and Staut (Ed.), *Nucleic Acid Chemistry: Improved and New Synthetic Procedures, Methods and Techniques*, John Wiley & Sons, Inc., 1978). In a specific embodiment, a method for producing a ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 protein is provided, which method comprises growing the

cells harboring plasmids containing the nucleic acid fragment encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 under conditions whereby these zinc finger proteins are expressed by the cell; and recovering the expressed zinc finger protein.

The isolated nucleic acids encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 typically consists of at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides, or a full-length coding sequence. In another embodiment, the nucleic acids are smaller than 35, 200, or 500 nucleotides in length. Nucleic acids can be single or double stranded. Nucleic acids that hybridize to or are complementary to the foregoing sequences, in particular the inverse complement to nucleic acids that hybridize to the foregoing sequences (*i.e.*, the inverse complement of a nucleic acid strand has the complementary sequence running in reverse orientation to the strand so that the inverse complement would hybridize without mismatches to the nucleic acid strand) are also provided. In specific aspects, nucleic acids are provided which comprise a sequence complementary to (specifically are the inverse complement of) at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 coding sequence.

The nucleic acids encoding ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 provided herein include those with nucleotide sequences encoding substantially the same amino acid sequences as found in Figures 6, and those encoding amino acid sequences with functionally equivalent amino acids.

F. ZFPm1, ZFPm2, ZFPm3, ZFPm4 and ZFPAp3 proteins

In yet another aspect, the present invention is directed to a zinc finger protein that is ZFPm1 (SEQ ID NO:38), ZFPm2 (SEQ ID NO:39), ZFPm3 (SEQ ID NO:40), ZFPm4 (SEQ ID NO:41) or ZFPAp3 (SEQ ID NO:42), preferably in combination with positive and negative regulating domains. The ZFPm1, ZFPm2, ZFPm3, and ZFPm4 zinc fingers are specific for the MIPS gene, meaning they can specifically and strongly bind to nucleotide sequence within the MIPS gene. ZFPAp3 is designed to bind to AP3 in Arabidopsis. The positive regulatory domain VP64 (Beerli *et al.*, *Proc. Natl. Acad. Sci. USA* (1998) 95:14628-14633) is fused to the C-terminal of each zinc finger domain. The negative regulatory domains SID (mSin3 interaction domain) (Ayer *et al.*, *Mol. Cell.*

Biol. (1996) 16:5772-5781) or SKD (a modified Kruppel-associated box) (Margolin *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91:4509-4513) are fused to the N-terminal of each zinc finger domain.

In yet another aspect, the present invention is directed to a zinc finger protein comprising a zinc finger nucleic acid binding domain and an effector domain, wherein said effector domain comprises an active domain of a restriction enzyme, an active domain of a nucleic acid modifying protein, *e.g.*, a nucleic acid methylase, a label or a modification.

The zinc finger proteins can be made by any methods known in the art. The zinc finger proteins can be produced by chemical synthesis (*see e.g.*, *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Chan and White (Ed.), Oxford University Press, 2000; *Peptide Synthesis Protocols*, Vol. 35, Pennington and Dunn (Ed.), Humana Press, 1995; and *Chemical Approaches to the Synthesis of Peptides and Proteins*, Lloyd-Williams *et al.* (Ed.), CRC Press, Inc., 1997), recombinant production (See generally, *Current Protocols in Molecular Biology* (1998) § 20, John Wiley & Sons, Inc.), or a combination thereof. Preferably, the zinc finger proteins are produced by recombinant production.

Functional fragments, analogs or derivatives of the ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 polypeptides are also provided. Preferably, such fragments, analogs or derivatives can be recognized an antibody raised against a ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 polypeptide. Also preferably, such fragments, analogs or derivatives comprise an amino acid sequence that has at least 60% identity, more preferably at least 90% identity to the ZFPm1 (SEQ ID NO:38), ZFPm2 (SEQ ID NO:39), ZFPm3 (SEQ ID NO:40), ZFPm4 (SEQ ID NO:41) or ZFPAp3 (SEQ ID NO:42) polypeptide (See Figure 6).

G. Antibodies recognizing ZFPm1, ZFPm2, ZFPm3, ZFPm4 and ZFPAp3 proteins

An antibody that specifically binds to the ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 proteins is also provided. Antibodies, whether polyclonal or monoclonal antibodies, can be raised against the zinc finger proteins by any methods known in the art

(see e.g., *Antibody Production: Essential Techniques*, Delves, Wiley, John & Sons, Inc., 1997; *Basic Methods in Antibody Production and Characterization*, Howard and Bethell, CRC Press, Inc., 1999; and *Monoclonal Antibody Production Techniques and Applications: Hybridoma Techniques*, Schook, Marcel Dekker, 1987). These antibodies can be used to assess the expression level and localization of ZFP protein in cells, e.g., plant cells.

For production of the antibody, various host animals can be immunized by injection with the ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 proteins, or a derivative of the foregoing, such as a cross-linked zinc finger protein. Such host animals include but are not limited to rabbits, mice, rats, and the like. Various adjuvants can be used to increase the immunological response, depending on the host species, and include but are not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lyssolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as bacille Calmette-Guerin (BCG) and corynebacterium parvum.

For preparation of monoclonal antibodies directed towards ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 proteins or domains, derivatives, fragments or analogs thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. Such techniques include but are not restricted to the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., *Immunology Today* 4:72 (1983)), and the EBV hybridoma technique to produce human monoclonal antibodies (Cole et al., *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). In an additional embodiment, monoclonal antibodies can be produced in germ-free animals (WO89/12690). Human antibodies may be used and can be obtained by using human hybridomas (Cote et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030 (1983)) or by transforming human B cells with EBV virus *in vitro* (Cole et al., *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). Techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Neuberger et al., *Nature* 312:604-608 (1984); and Takeda

et al., *Nature* 314:452-454 (1985)) by splicing the genes from a mouse antibody molecule specific for the ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 protein together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

5 Techniques described for the production of single chain antibodies (U.S. patent 4,946,778) can be adapted to produce ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 protein-specific single chain antibodies. An additional embodiment utilizes the techniques described for the construction of Fab expression libraries (Huse et al., *Science* 246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab

10 fragments with the desired specificity for ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 proteins, or domains, derivatives, or analogs thereof. Non-human antibodies can be "humanized" by known methods (see, e.g., U.S. Patent No. 5,225,539).

15 Antibody fragments that contain the idiotypes of ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 proteins can be generated by techniques known in the art in accordance with the methods of the present invention. For example, such fragments include but are not limited to: the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of the F(ab')2 fragment, the Fab fragments that can be generated by treating the antibody molecular with papain and a reducing agent, and Fv fragments.

20 In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art in accordance with the methods of the present invention, e.g., ELISA (enzyme-linked immunosorbent assay). To select antibodies specific to a particular domain of the ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 proteins, one may assay generated hybridomas for a product that binds to the fragment of a ZFPm1, ZFPm2, ZFPm3, ZFPm4 or ZFPAp3 proteins that contain such a domain.

H. Nucleic acids encoding ZFP-SID fusion proteins

30 In yet another aspect, the present invention is directed to an isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a fusion protein comprises a zinc finger domain, e.g., 2C7 and an effector domain of SID (mSin3A Interaction

Domain). A fusion protein comprising the 2C7 and the SID domains is termed as a 2C7-SID fusion protein. The isolated nucleic acid fragment can be DNA or RNA. An isolated nucleic acid fragment, which is hybridizable to the nucleic acid fragment encoding the 2C7-SID fusion protein under low, medium and high stringency condition is also provided. Preferably, the isolated nucleic acid fragment hybridizes to the nucleic acid fragment encoding the 2C7-SID fusion protein under high stringency condition. Also preferably, the isolated nucleic acid fragment has the nucleotide sequence set forth in SEQ ID NO:5 or SEQ ID NO:66. Plasmids comprising the nucleic acid fragment encoding the 2C7-SID fusion protein and cells comprising such plasmids are further provided. Any suitable cells can be used and preferably, bacterial cells, yeast cells, fungal cells, plant cells, insect cells and animal cells are used.

The nucleic acid fragment encoding the 2C7-SID fusion protein can be prepared by any methods known in the art, e.g., recombinant production, chemical synthesis or a combination thereof (See generally, *Current Protocols in Molecular Biology* (1998) § 20, John Wiley & Sons, Inc; Knorre, *Design and Targeted Reactions of Oligonucleotide Derivative*, CRC Press, 1994; and Staut (Ed.), *Nucleic Acid Chemistry: Improved and New Synthetic Procedures, Methods and Techniques*, John Wiley & Sons, Inc., 1978). In a specific embodiment, a method for producing a 2C7-SID fusion protein is provided, which method comprises growing the cells harboring plasmids containing the nucleic acid fragment encoding the 2C7-SID fusion protein under conditions whereby these zinc finger proteins are expressed by the cell; and recovering the expressed 2C7-SID fusion protein.

The isolated nucleic acids encoding the 2C7-SID fusion protein typically consists of at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides, or a full-length coding sequence. In another embodiment, the nucleic acids are smaller than 35, 200, or 500 nucleotides in length. Nucleic acids can be single or double stranded. Nucleic acids that hybridize to or are complementary to the foregoing sequences, in particular the inverse complement to nucleic acids that hybridize to the foregoing sequences (*i.e.*, the inverse complement of a nucleic acid strand has the complementary sequence running in reverse orientation to the strand so that the inverse complement would hybridize without mismatches to the nucleic acid strand) are also

provided. In specific aspects, nucleic acids are provided which comprise a sequence complementary to (specifically are the inverse complement of) at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of the 2C7-SID fusion protein coding sequence.

5 The nucleic acids encoding the 2C7-SID fusion protein provided herein include those with nucleotide sequences encoding substantially the same amino acid sequences as found in Figures 6 and 24, and those encoding amino acid sequences with functionally equivalent amino acids.

10 Any suitable SID domains can be used. Preferably, the SID domain is derived from a SID domain of the MAD 1 protein.

I. ZFP-SID fusion proteins

In yet another aspect, the present invention is directed to a ZFP-SID, e.g., 2C7-SID, fusion protein comprising a zinc finger of 2C7 and an effector domain of SID. The negative regulatory domains SID (mSin3 interaction domain) (Ayer *et al.*, *Mol. Cell. Biol.* (1996) 16:5772-5781) or SKD (a modified Kruppel-associated box) (Margolin *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91:4509-4513) can be fused to the C- or N-terminal of the 2C7 zinc finger domain. Preferably, the SID domain is fused to the N-terminal of the 2C7 zinc finger domain. Also preferably, the 2C7-SID fusion protein comprises a nuclear localization signal.

The 2C7-SID fusion protein can be made by any methods known in the art. It can be produced by chemical synthesis (*see e.g.*, *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Chan and White (Ed.), Oxford University Press, 2000; *Peptide Synthesis Protocols*, Vol. 35, Pennington and Dunn (Ed.), Humana Press, 1995; and *Chemical Approaches to the Synthesis of Peptides and Proteins*, Lloyd-Williams *et al.* (Ed.), CRC Press, Inc., 1997), recombinant production (See generally, *Current Protocols in Molecular Biology* (1998) § 20, John Wiley & Sons, Inc.), or a combination thereof. Preferably, the 2C7-SID fusion proteins are produced by recombinant production. Functional fragments, analogs or derivatives of the 2C7-SID fusion proteins or polypeptides are also provided. Preferably, such fragments, analogs or derivatives can be recognized an antibody raised against a 2C7-SID fusion protein or polypeptide. Also

preferably, such fragments, analogs or derivatives comprise an amino acid sequence that has at least 60% identity, more preferably at least 90% identity to the 2C7-SID fusion protein encoded by the nucleotide sequence set forth in SEQ ID NO:5 or SEQ ID NO:66.

5 **J. Antibodies recognizing ZFP-SID fusion protein**

An antibody that specifically binds to a ZFP-SID, e.g., 2C7-SID, fusion proteins is also provided. Antibodies, whether polyclonal or monoclonal antibodies, can be raised against the 2C7-SID fusion proteins by any methods known in the art (*see e.g., Antibody Production: Essential Techniques*, Delves, Wiley, John & Sons, Inc., 1997; *Basic Methods in Antibody Production and Characterization*, Howard and Bethell, CRC Press, Inc., 1999; and *Monoclonal Antibody Production Techniques and Applications: Hybridoma Techniques*, Schook, Marcel Dekker, 1987). These antibodies can be used to assess the expression level and localization of ZFP protein in cells, e.g., plant cells.

For production of the antibody, various host animals can be immunized by injection with the 2C7-SID fusion proteins, or a derivative of the foregoing, such as a cross-linked fusion protein. Such host animals include but are not limited to rabbits, mice, rats, and the like. Various adjuvants can be used to increase the immunological response, depending on the host species, and include but are not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lyssolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as bacille Calmette-Guerin (BCG) and corynebacterium parvum.

For preparation of monoclonal antibodies directed towards 2C7-SID fusion proteins or domains, derivatives, fragments or analogs thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. Such techniques include but are not restricted to the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., *Immunology Today* 4:72 (1983)), and the EBV hybridoma technique to produce human monoclonal antibodies (Cole et al., *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). In an additional embodiment, monoclonal antibodies can be produced

in germ-free animals (WO89/12690). Human antibodies may be used and can be obtained by using human hybridomas (Cote et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030 (1983)) or by transforming human B cells with EBV virus *in vitro* (Cole et al., *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)).

5 Techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Neuberger et al., *Nature* 312:604-608 (1984); and Takeda et al., *Nature* 314:452-454 (1985)) by splicing the genes from a mouse antibody molecule specific for the 2C7-SID fusion proteins together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies

10 are within the scope of this invention.

Techniques described for the production of single chain antibodies (U.S. patent 4,946,778) can be adapted to produce 2C7-SID fusion protein-specific single chain antibodies. An additional embodiment utilizes the techniques described for the construction of Fab expression libraries (Huse et al., *Science* 246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for 2C7-SID fusion proteins, or domains, derivatives, or analogs thereof. Non-human antibodies can be "humanized" by known methods (*see, e.g.*, U.S. Patent No. 5,225,539).

15 Antibody fragments that contain the idiotypes of 2C7-SID fusion proteins can be generated by techniques known in the art in accordance with the methods of the present invention. For example, such fragments include but are not limited to: the F(ab')² fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of the F(ab')² fragment, the Fab fragments that can be generated by treating the antibody molecular 20 with papain and a reducing agent, and Fv fragments.

25 In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art in accordance with the methods of the present invention, *e.g.*, ELISA (enzyme-linked immunosorbent assay). To select antibodies specific to a particular domain of the 2C7-SID fusion proteins, one may assay generated hybridomas for a product that binds to the fragment of a 2C7-SID fusion protein that contains such a domain.

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All references cited herein are incorporated by reference as if each were specifically incorporated by reference.

The following examples are intended to illustrate but not to limit the invention.

25 **K. EXAMPLES**

The invention is illustrated by materials and methods for controlling genetic expression in plants, e.g., the luciferase transgene, Arabidopsis AP3 gene, and the maize MIP5 gene. "Providing" herein means maintaining a plant cell, either in planta or ex planta, either in a plant or in cell or tissue culture, under conditions in which expression and production of a desired zinc finger protein is produced in the plant cell. The regulatory factor, or "effector protein," will at least comprise a zinc finger component

which binds specifically to a nucleotide sequence contained within the target gene such that the binding of the zinc finger protein modulates expression of the target gene (See generally Kim and Pabo, *Proc. Natl. Acad. Sci. U.S.A.*, 95:2812-2817 (1998)) and may also contain a regulatory domain. Thus, the zinc finger protein can be fused to an additional amino acid sequence which provides regulation, although as stated above, in some instances, binding of the zinc finger portion alone has an effect on expression. This is especially the case when the binding target is in the TATTA box region where transcription is initiated.

10 Example 1

ZFP-effector fusion protein function on luciferase reporter gene in maize cells

To establish this Zinc Finger Protein (“ZFP”) technology in plants, we have shown that transient trans-activation and trans-repression of a reporter gene can be achieved in representative plant cells. These transient regulation is mediated by a previously synthesized synthetic zinc finger proteins, 2C7. The binding site of zinc finger protein 2C7 sequence was originally from the type 1 human immune deficiency virus (HIV-1) genome. The synthetic zinc finger protein 2C7 has been show to be able to specifically bind to this site and activate reporter gene’s expression in human Hela cell line (Liu *et al.*, *Proc. Natl. Acad. Sci. USA* (1997) 94:5525-5530; and Wu *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:344-348). The claimed method comprises providing plant cells, either in culture or in intact plants, with a functional ZFP, or an expression system for production of a suitable ZFP. However, by “culturing” is meant maintaining a plant cell, either in planta or ex planta, either in a plant or in cell or tissue culture, under conditions in which expression and production of a desired zinc finger protein is produced in the plant cell.

25 A. Reporter constructs

Luciferase reporter plasmids were constructed from the Cassava Vein Mosaic Virus promoter (CsVMV) (SEQ ID NO:1) (Calvert *et al.*, *J. Gen. Virol.* (1995) 76:1271-1278; Verdaguer *et al.*, *Plant Mol. Biol.* (1996) 31:1129-1139, Verdaguer *et al.*, *Plant Mol. Biol.* (1998) 37:1055-1067). Six tandem repeats of 2C7 binding sites (6X2C7)

(SEQ ID NO:2) was inserted at the upstream of reporter. Reporter I (p5'C7F) is a deleted and thus inactive version of promoter CsVMV which contains nucleotide sequence -112 bp to +72 bp of the full length CsVMV promoter with 6X 2C7 binding site is inserted at the 5' end. Reporter II (pc7rbTATA) contains a minimal promoter (SEQ ID NO:3) with 6X2C7 binding site inserted at the 5' end. Reporter I contains a longer promoter sequence and is stronger than the promoter in reporter I (Fig. 1).

5 B. Effector constructs

Maize ubiquitin promoter ZmUbi (Cornejo *et al.*, *Plant Mol Biol* (1993) 23(3):567-81) was used to express the ZFP-effector fusion proteins. The activation construct (pND3008) (SEQ ID NO:4) consists of several functional domains: zinc finger protein domain 2C7, nuclear localization signal, the transcriptional activation domain VP64 which is fused to the relative C-terminal of ZFP2C7 and HA epitope tag sequence (Fig. 1). The function of HA tag is for the detection of zinc finger protein expression through Western procedures. The repression construct (pND3018) (SEQ ID NO:5) consists of repression domain SID, nuclear localization signal, ZFP2C7 domain, and HA epitope tag sequence. The repression domain SID is fused to the relative N-terminal of ZFP2C7 domain.

10 C. Transient assay

15 Maize protoplasts were prepared from maize cell line HE89 (F19556) and transformed using standard procedure (Chourey and Zurawski, *Theor. Appl. Genet.* 59:341-344 (1981)). For each transformation reaction, 1ug of luciferase reporter constructs and 10ug activator were co-transformed into one million purified protoplasts. The transformed protoplasts were incubated at 28°C in dark for 24 - 48 hrs. Collect cells by centrifugation at 500g for 5min. Aspirate off media. Resuspend in 80ul 1.2x Passive Lysis Buffer (Promega). Freeze at -80°C for >10min. Thaw completely at room temp. Vortex and spin at 3500RPM for 5min at 4°C. Collect supernatant. Assay 20ul extract for luciferase with the Luciferase Assay Kit (Promega).

20 The luciferase activity of the target reporter is normalized to the protein contents of each sample to generate the specific activity. This will correct for any variations in transformation efficiency and cell extractions.

D. Function of ZFP-effector fusion protein in maize protoplasts

5

The effects of the 2C7-VP64 ZFP-activator fusion was tested on Reporter I and II reporters (Fig. 1). With Reporter I (p5'c7F), 146 to 250 fold of activation is achieved with the 2C7-Vp64 activator. With Reporter II (pc7rbTATA), 40 to 70 fold of activation is achieved with the 2C7-Vp64 activator.

10 The effect of the 2C7-SID ZFP-repressor fusions on tested on Reporter I. The 2C7-SID effector produces almost 2 fold of repression.

Example 2

10 Position effect of zinc finger protein binding site on the activation of luciferase reporter constructs in tobacco cells

A. Reporter constructs

15 A series of luciferase reporter plasmids were constructed from the full length and partial deletion version of Cassava Vein Mosaic Virus promoter (CsVMV promoter with six tandem repeats of 18bp 2C7 (GCG TGG GCG GCG TGG GCG) (SEQ ID NO:43) binding sites (6X2C7) inserted at different locations within fragments of this promoter (Fig. 2) (Verdaguer *et al.*, *Plant Mol. Biol.* (1998) 37:1055-1067). Plasmid pAluc contains a full-length CsVMV promoter (same as in pND3008, see SEQ ID NO:1) driving the expression of luciferase gene. Plasmid p5'C7A contains the same full-length CsVMV promoter with 6X2C7 inserted at the 5' end using Xba I site. Plasmid p3'C7A is almost identical to p5'C7A except the 6X2C7 was inserted at the 3' end using Xba I site. Plasmid pC7δE contains the deleted CsVMV promoter with 6X2C7 binding site replacing nucleotide sequence -112 to -63 of CsVMV. Plasmid p5'C7C contains a partial deleted CsVMV (-222 to +72) with 6X2C7 inserted at the 5' end. Plasmid p5'C7D contains a partial deleted CsVMV (-178 to +72) with 6X2C7 inserted at the 5' end. Plasmid p5'C7F and pc7rbTATA are the same in example 1A. Plasmid prbTATA is identical to pc7rbTATA except no 6X2C7 binding site. The promoter activity in each construct is further decreased than the previous version as the promoter sequence gets shorter. For example, the activity of p5'C7A is higher than p5'C7C, p5'C7D is higher than p5'C7F, and their activities are all higher than the minimal promoter construct pc7rbTATA.

B. Effector constructs

Tobacco mosaic virus (TMV) (Gibbs, *CMI/AAB Descriptions of plant viruses* (1977) 184) was used as an expression vector for the ZFP-effector fusion proteins. The coding region for the coat protein was replaced by the ZFP fusion sequence (Fig. 3).
5 Plasmid p2C7-SID consists of TMV promoter and SID-2C7 repression domain (same as in pND3018). Plasmid p2C7-VP64 consists of TMV promoter and 2C7-VP64 activation domain (same as in pND3008). Plasmid pC7-VP64 is similar to p2C7-VP64 except the 6 finger zinc finger protein 2C7 (in pND3008 and pND3018) is replaced by 3 finger protein C7 (SEQ ID NO:6). Plasmid pC7-GFP consists TMV promoter and GFP fragment (Ref).
10 It was used as background control. Arabidopsis ubiquitin promoter UBQ3 was also used to express the ZFP fusion proteins(constructs did not show here and the results are similar).

C. Transient assay

TMV viral RNA was made from the effector constructs with the T7 Megascript Kit (Ambion). Collect log phase tobacco BY-2 cells in conical tubes. Wash with 0.4M mannitol. Digest cell walls by adding 20 ml Enzyme Solution (1% Cellulase Onezuka RS (Karlan Research USA #2019), 0.1% Pectolyase Y-23 (Karlan Research USA #8006), 0.4M mannitol, pH 5.8). Incubate in deep petri dish (NUNC 4031) at 22-28°C in dark with continuous or occasional swirling for 2hrs. Wash 2x with 0.4M mannitol. Collect by centrifugation at 300g for 2min. Remove as much supernatant as possible and resuspend in 15-25ml Electroporation Buffer (0.3M mannitol, 5mM MES, 70mM KCl, pH 5.8). Count cells on hemacytometer and adjust concentration to 1.6×10^6 /ml. Add 0.3ml cells to a 0.4cm electroporation cuvette. Add 100ul Electroporation Buffer containing 1ug reporter plasmid DNA, 0.1ug/ml Renilla Luciferase (RL) control plasmid with no ZFP binding sites, and 25ug viral RNA, mix gently with pipette, and immediately electroporate ($R=\infty$, $C=125\mu F$, $V=300V$). Incubate on ice for 30min. Incubate at RT for 5min. Transfer 100ul electroporated cells to 3ml Protoplast Culture Media (2.2g/l Murashige and Skoog Plant Salt Base- Gibco BRL #11117-074, 0.1g/l myo-inositol, 1mg/l thiamin-HCl, 0.2mg/l 2,4-D, 10g/l sucrose, 0.4M mannitol, and pH 5.8). Incubate cells in 3ml media in 15ml conical tube placed on side or in 1-2ml media in a small petri dish (Corning #25050-35). Incubate at 28°C in dark for 48 hrs. Collect cells by

centrifugation at 500g for 5min. Aspirate off media. Resuspend in 80ul 1.2x Passive Lysis Buffer (Promega). Freeze at -80°C for >10min. Thaw completely at room temp. Vortex and spin at 3500RPM for 5min at 4°C. Collect supernatant. Assay 20ul extract for luciferase and RL activity with the Dual Luciferase Assay Kit (Promega).

5 In this transient assay, the luciferase activity of the target reporter is normalized to the RL activity of the control reporter by division. This will correct for any variations in transformation efficiency and cell extract concentration. The normalized activity of an effector is presented as the normalized activity of reporter plus effector relative to the activity of the target reporter without effector expression. Thus, a relative activity of 1
10 means there is no effector function.

D. Effect of binding site position and ZFP-activator fusion protein function in tobacco protoplasts

The effects of the C7-VP64 and 2C7-VP64 ZFP-activator fusions were tested on these reporters (Fig. 4). The results show zinc finger domain 2C7-VP64 fusion protein activated the expression of luciferase reporter gene in both tobacco cells and in maize cells. The activation level was dependent on the position of zinc finger protein binding sites. Generally, the closer of the binding site is to the TATA box, the higher of the activation level. Three-finger zinc finger protein (C7) generally gives higher activation level than the six-finger zinc finger protein (2C7). It is probably due to the fact of doubling the total numbers of activator when a 3-finger protein was used. With a minimal promoter, Reporter II (pc7rbTATA), almost 100-fold of activation is achieved with the C7-Vp64 activator. The 2C7-VP64 activator gives 23-fold of activation. A stronger promoter, p5'c7F (Reporter I), is activated 10 fold by C7-VP64 and 5 fold by 2C7-VP64. From this experiment, it is clear that the zinc finger-binding site is preferred upstream of
25 TATA box.

E. Effect of ZFP-repressors in tobacco protoplasts

To determine whether repression can be achieved, the effects of the 2C7-SID, 2C7-SKD, and e2c-SID ZFP-repressor fusions on three different reporter plasmids: Reporter I (p5'C7F), Reporter II (pc7rbTATA), and pC78E. Where studied. The 2C7-SID effector produces almost 5-fold repression of all the reporters (Fig. 5). The 2C7-

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SKD shows no specific repression of any of the reporters. Similarly, the e2c-SID repressor does not cause much repression of the reporters (Fig. 5).

Thus, ZFP fusions to the SID repressor domain provide effective, specific repression of their target genes. However, the repression domain SKD and the e2c zinc finger protein are not functional in plant cells.

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Example 3

Selection of zinc finger protein binding sites for endogenous gene MIPS and AP3

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The genes for AP3 (U30729) and MIPS (AF056326) were examined for suitable zinc finger binding sites. At the time of construction, the zinc finger library covered all the GNN triplets and the TGA triplet as shown in the recognition code provided in Table 2) (see also Segal *et al.*, *Proc. Natl. Acad. Sci. USA* (1999) 96:2758-2763. Potential zinc finger-binding sites were selected by searching for 6 triplets (18 consecutive base pairs) with either GNN or TGA.

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Table 2. GNN triplets and the TGA triplet recognition code

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	<u>Target Sequence</u>	<u>ZFP domain</u>
	GAA	SQSSNLV (SEQ ID NO:44)
	GAC	SDPGNLV (SEQ ID NO:45)
	GAG	SRSDNLVR (SEQ ID NO:46)
	GA	STSGNLV (SEQ ID NO:47)
	GCA	SQSGDLRR (SEQ ID NO:48)
	GCC	SDCRDLAR (SEQ ID NO:49)
	GCG	SRSDDLVR (SEQ ID NO:50)
	GCT	STSGELV (SEQ ID NO:51)
	GGA	SQSSHDLVR (SEQ ID NO:52) or SQRAHLER (SEQ ID NO:53)
	GGC	SDPGHLVR (SEQ ID NO:54)
	GGG	SRSDKLVR (SEQ ID NO:55)
	GGT	STSGHLVR (SEQ ID NO:56)

	GTA	SQSSSLV R (SEQ ID NO:57)
	GTC	SDPGALV R (SEQ ID NO:58)
	GTG	SRSDVLV R (SEQ ID NO:59) or SRKDSLVR (SEQ ID NO:60)
5	GTT	STSGSLV R (SEQ ID NO:61)
	TGA	SQAGHLAS (SEQ ID NO:62)

For the AP3 gene, only one target site was identified as a possible ZFP binding site using the current code. This site (5'-tac ttc ttc aac tcc atc-3') was located from -112 to -95 relative to the start of translation, about 70 base pairs upstream of the start of transcription. ZFPAp3 was selected to bind the compliment of this sequence (5'-GAT GGA GTT GAA GAA GTA-3') (SEQ ID NO:7). Of course, with a complete code falls in place in combo with present leadings, any sequence can be targeted.

For the MIPS gene, there were two possible regions in which ZFP could be targeted using current code. One was at the very 5' end of the cDNA from position -85 to -65 relative to the translational start site (5'-GCC TCC TTC CTC CTC TCA CTC-3') (SEQ ID NO:8). Two possible zinc fingers could be made to this site. ZFPm1 binds the compliment of this sequence from -68 to -85 (5'- TGA GAG GAG GAA GGA GGC-3') (SEQ ID NO:9) and ZFPm2 binds from -65 to -82 (5'- GAG TGA GAG GAG GAA GGA-3') (SEQ ID NO:10) of the compliment. Another location for ZFP binding sites was within the translated region of the gene from 294 to 317 (5'-GCC AAC TAC TAC GGC TCC CTC ACC-3') (SEQ ID NO:11) after the translational start site. Two ZFPs were selected to bind the compliment of this site. ZFPm3 binds from 311 to 294 (5'- GGA GCC GTA GTA GTT GGC-3') (SEQ ID NO:12) and ZFPm4 binds from 317 to 300 (5'- GGT GAG GGA GCC GTA GTA-3') (SEQ ID NO:13). All four of these zinc finger proteins of the six-finger (hectadactyl) type were constructed. The activity of these different zinc fingers when fused to transcriptional regulatory domains should provide us with more information about the optimal binding position. This information will aid in selecting binding sites for new target genes.

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Example 4

Construction of new zinc finger protein ZFPAp3, ZFPm1, ZFPm2, ZFPm3, and ZFPm4

Since the specificity of the ZFP is determined by the DNA binding alpha helix, polydactyl zinc finger proteins with novel DNA specificity can be constructed by modifying the recognition helices of existing zinc finger proteins. A human zinc finger protein Sp1C has been selected to serve as a framework in the present example. It has been demonstrated that the Sp1C protein can provide a good framework for zinc finger domain modification (Beerli *et al.*, *Proc. Natl. Acad. Sci. USA* (1998) 95:14628-14633).

New zinc finger proteins were constructed in two steps. First, three-finger ZFPs are constructed by PCR from overlapping oligonucleotides. These three-finger ZFPs are then fused together to create a six-finger (polydactyl) zinc finger protein to bind a specific 18bp sequence. PCR construction of three-finger proteins was carried out in two sequential PCR reactions. In PCR1, the F2-b (finger 2-backward) and F2-f (finger 2-forward) primers were used as a template for PCR extension reaction with the F1-f1 (finger 1- first forward) and F3-b1 (finger 3- first backward) primers.

For example, in a 100ul reaction, 1ug F1-f1, 1ug F3-b1, 0.1ug F2-f, 0.1ug F2-b, 8ul 2.5mM dNTP mix, 10ul 10x Taq Buffer with 15mM MgCl₂ (Perkin Elmer), 0.5ul 5u/l AmpliTaq Gold (Perkin Elmer). PCR conditions 1) 94°C for 30sec; 2) 94°C for 30sec; 3) 60°C for 30sec; 4) 72°C for 30sec; 5) repeat steps 2-4, 25 times; 6) 72°C for 1min. In the second reaction, PCR2, 1ul of the PCR extension product from PCR1 is used as a template for PCR with F1-f2 (finger 1 second forward) and f3-b2 (finger 3 second backward) to extend the construct. In a 100ul reaction, 1ug F1-f2, 1ug F3-b2, 1ul PCR1 reaction, 8ul 2.5mM dNTP mix, 10ul 10x Taq Buffer with 15mM MgCl₂ (Perkin Elmer), 0.5ul 5u/l AmpliTaq Gold (Perkin Elmer). Amplify with same conditions used in PCR1. The 320bp PCR product was digested with SfiI and the 300bp fragment was isolated and ligated into a derivative of the pMal-C2 vector (New England Biolabs). When transformed into XL1-Blue *E. coli*, this vector provided IPTG-inducible expression of the zinc finger fused to the Maltose Binding Protein (MBP). The MBP fusion allows easy purification and detection of the zinc finger protein. Similarly, the second half of zinc finger protein domain (also containing three fingers) was synthesized using PCR extension reaction.

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The following provides details of a typical construction of a zinc finger, in this case, construction of ZFPm2: The following primers were used to construct the ZFPm2a protein: F1-f1 (ggt aag tcc ttc AGC CGC AGC GAT AAC CTG CTG CGC cac cag cgt acc cac acg ggt gaa aaa ccg tat aaa tgc cca gag), F1-f2 (gag gag gag gag gtg gcc cag ggc gcc ctc gag ccc ggg gag aag ccc tat gct tgt ccg gaa tgt ggt aag tcc ttc AGC CGC AGC), F2-f (GC CAG GCC GGC CAC CTG GCC AGC cat caa cgc act cat act ggc gag aag cca tac aaa tgt cca gaa tgt ggc), F2-b (GCT GGC CAG GTG GCC GGC CTG GCT aaa aga ttt gcc gca ctc tgg gca ttt ata egg ttt ttc acc), F3-b1 (CCG GAC GAG ATT GTC AGA CCG AGA gaa aga ctt gcc aca ttc tgg aca ttt gta tgg c), F3-b2 (gag gag gag gag ctg gcc ggc ctg gcc act agt ttt acc ggt gtg agt acg ttg gtg CCG GAC GAG ATT GTC AGA CCG). These primers were used as described above to generate the 3-finger protein, ZFPm2a (SEQ ID NO:63):

1	gaggaggagg aggtggccca ggccgcctc gagcccggg agaagcccta	50
51	tgcttgtccg gaatgtggta agtccttcAG CCGCAGCGAT AACCTGGTGC	100
101	GCCaccagcg tacccasacg ggtaaaaac cgtataaatg cccagagtgc	150
151	ggcaaattt ttAGCCAGGC CGGCCACCTG GCCAGCcatc aacgcactca	200
201	tactggcgag aagccataca aatgtccaga atgtggcaag tcittcTCTC	250
251	GGTCTGACAA TCTCGTCCGG caccaacgta ctcacaccgg taaaaaaaaact	300
301	agtggccagg ccggccagct ctcctccctc	330

This protein has the framework of the Sp1C zinc finger protein (lower case sequences) with the DNA recognition helices replaced with the appropriate sequences (upper case sequences) to generate a zinc finger with a new binding specificity (5'-GAG TGA GAG). A similar set of primers was used to generate the ZFPm2b (SEQ ID NO:64) protein:

1	gaggaggagg aggtggccca ggccgcctc gagcccggg agaagcccta	50
51	tgcttgtccg gaatgtggta agtccttcTC TCAGAGCTCT CACCTGGTGC	100
101	GCCaccagcg tacccacacg ggtaaaaac cgtataaatg cccagagtgc	150
151	ggcaaattt ttAGCCAGTC CAGAACCTG GTGCGCcatc aacgcactca	200
201	tactggcgag aagccataca aatgtccaga atgtggcaag tcittcTCTC	250
251	GGTCTGACAA TCTCGTCCGG caccaacgta ctcacaccgg taaaaaaaaact	300

301 agtggccagg ccggccagct cctccctc

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This protein also has a Sp1C framework with altered recognition helices to bind (5'-GAG GAA GGA-3'). The first set of three finger protein (ZFPa) was fused to C-terminal to 5 the second set of three finger protein (ZFPb) by ligating the ZFPa SpeI/XbaI 0.3kb fragment and ZFPb XbaI-BsrFI 0.3kb fragment into the pMal-C2 vector digested with XbaI and SpeI. The resulting six-finger zinc finger protein should specifically bind the target 18 bp sequence 5'-GAG TGA GAG GAG GAA GGA-3' (SEQ ID NO:65).

Using the same method, three-finger proteins with a Sp1C framework were made 10 to bind the m1, m3, m4, and Ap3 target sites. These three-finger proteins were fused together to create the six-finger zinc finger. Restriction sites were designed in to allow ligation of the fragments. The five constructs that carried the ZFP genes were named as 15 pMAL-m1, pMAL-m2, pMAL-m3, pMAL-m4, and pMAL-Ap3. The coding regions are the 500 bp fragment from Sfi digestion of each pMal plasmid and are named ZFPm1 (SEQ ID NO:14), ZFPm2 (SEQ ID NO:15), ZFPm3 (SEQ ID NO:16), ZFPm4 (SEQ ID NO:17), and ZFPAp3 (SEQ ID NO:18).

Example 5

Expression and Purification of ZFPAp3, ZFPm1, ZFPm2, ZFPm3, and ZFPm4 proteins
in *E. coli*

A. Preparation of zinc finger protein crude extracts

Zinc Finger Protein fusions were prepared as follows, primarily to obtain material for use in raising anti-ZFP antibodies and to assess binding affinity and specificity in vitro.

25 *E. coli* (XL1-Blue and (K12TB1) containing the zinc finger expression plasmid was first collected for each construct. Grow 3ml culture of bacteria overnight in SB (10g/l MOPS, 30g/l Bacto Peptone, 20g/l Yeast extract) + 50ug/ml Carbenicillin + 1% Glucose at 37°C. Start 5ml culture w/ 0.25ml of overnight culture in SB + 50ug/ml Carbenicillin + 0.2% Glucose + 90um ZnCl₂ at 37°C for 2hrs. Add IPTG to a concentration of 0.3mM. Shake at 37°C for 2hrs. Pellet bacteria at 2000g for 5min.

Resuspend pellet in 0.3ml Zn Buffer A (10mM Tris base, 90mM KCl, 1mM MgCL₂, pH 7.5) +5mM DTT. Freeze suspension at -80°C. Freeze thaw 6 times between dry ice/ethanol bath and 37°C water bath. Spin 14K for 5min at 4°C. The supernatant is then ready for ELISA assay.

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B. Purification of ZFPAp3, ZFPm1, ZFPm2, ZFPm3, and ZFPm4

All five zinc-finger proteins were affinity purified using New England Biolab's pMAL protein fusion purification Kit. One hundred ml of *E. coli* (K12TB1) that carried construct pMAL-m1, pMAL-m2, pMAL-m3, pMAL-m4, and pMAL-Ap3 separately was grown at 30°C for overnight under the selection of Carbenicillin (same condition as above). Cells were collected through centrifugation and lysed through brief sonication on ice. The ZFPm1-MBP fusion protein was purified from this total lysate using New England Biolab's Kit (#800). The purified samples were quantitated and loaded on SDS-PAGE to estimate the quality. All five proteins were shown as single band on SDS-PAGE gel.

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C. ZFP antibody production

Two antibodies were generated from the purified zinc finger proteins. Antibody I was made against the ZFPm1 antigen. Antibody II was made against an equal mixture of the ZFPm3 and ZFPm4 antigens. Both antibodies were titrated using ELISA procedure and Western Blots of zinc finger protein expressing cells.

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Example 6

Characterization of DNA binding specificity of ZFPAp3, ZFPm1, ZFPm2, ZFPm3, and ZFPm4

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Zinc finger protein can be characterized by any methods known in the art. For example, the zinc finger protein can be characterized by *in vitro* assay such as ELISA and *in vivo* assay such as gel shifting assay. ELISA assay can be used to characterize binding specificity of a particular zinc finger protein for a variety of target nucleotide sequences. Gel shifting assay can be used to characterize binding affinity, *i.e.*, obtaining binding constant, of a particular zinc finger protein for a particular target nucleotide sequence.

In this study, crude extracts of the three-finger and six-finger proteins were used for ELISA assay to evaluate the newly synthesized zinc finger protein's DNA binding specificity. The assay was also repeated with the purified six-finger proteins.

A. ELISA procedures

5 Coat ELISA plates (Costar 3690) with streptavidin. Use 25ul/well of 8ug/ml streptavidin in PBS. Incubate at 37°C for 1hr. Wash 2x with dH2O. Bind wells with 25ul/well of 4ng/ul biotinylated target oligonucleotide in PBS. Incubate at 37°C for 1hr. Wash 2x with dH2O. Block with 175ul/well of 3% BSA in Zn Buffer A. Incubate at 37°C for 1hr. Tap plates dry. Perform 1:2 serial dilutions of extract in Binding Buffer 10 (1%BSA, 5mM DTT, .12ug/ul Salmon Sperm DNA in ZnBA). Usually from 1:4 to 1:512 for multi-target ELISA or 1:4 to 1:32 for screening clones for specific DNA binding activity. Incubate 1hr at room temperature. Wash 8x with dH2O. Add primary antibody: 25ul/well of 1:1000 dilution of mouse anti-MBP (Sigma M-6295) in 1%BSA 15 ZnBA (no DTT). Incubate 30min at room temperature. Wash 8x with dH2O. Add secondary antibody: 25ul/well of 1:1000 dilution of goat anti-mouse/Alkaline Phosphatase (Pierce 31324) in 1%BSA ZnBA (no DTT). Incubate 30min at room temperature. Wash 8x with dH2O. Develop with 25ul developer (1 tablet of Sigma 104 Phosphatase Substrate (Sigma 104-105)/ 5ml AP Developing Buffer [100ml diethanolamine+0.05g MgCl₂ + 97.5 Sodium Azide, adjust to 500ml with ddH₂O, pH 20 9.88]). Incubate at room temperature for 5-60min until color reaction is yellow. Read A405 with a plate reader.

B. ZFP specificity evaluation

Hairpin oligonucleotides with a 5' biotin conjugate were synthesized. The oligo m12 (5'-Biotin-GGa gcc tcc ttc ctc ctc tca ctc GGG TTTT CCC gag tga gag gag gaa gga 25 ggc tCC-3') (SEQ ID NO:19) has the target sites for ZFPm1 and ZFPm2. Oligo m34 (5'- Biotin-GGa gcc aac tac tac ggc tcc ctc acc GGG TTTT CCC ggt gag gga gcc gta gta gtt ggc tCC-3') (SEQ ID NO:20) has the target sites for ZFPm3 and ZFPm4. The oligo Ap3 has the target site for the ZFPAp3 protein (5'-Biotin-GGt tac ttc aac tcc atc GGG 30 TTTT CCC gat gga gtt gaa gaa gta aCC-3') (SEQ ID NO:21) . The individual three-finger ZFPs were evaluated on ELISA with the m12, m34, and Ap3 target oligos. In every case, the three-finger proteins bound their target oligo with significantly higher

affinity than the non-target oligos. The eight non-target oligos are: NRI-1 (SEQ ID NO:22), NRI-2 (SEQ ID NO:23), hHD-I (SEQ ID NO:24), hHD-II (SEQ ID NO:25), c5c1-g (SEQ ID NO:26), c5p3-g (SEQ ID NO:27), B3c2 (SEQ ID NO:28), and e2c-g (SEQ ID NO:29). All five six-finger proteins, ZFPm1, ZFPm2, ZFPm3, ZFPm4, and ZFPAp3 were tested more extensively by ELISA with the m12, m34, Ap3, and the above eight non-target oligos. The six-finger proteins bound their targets better than any of the non-target oligos. ZFPm1, ZFPm4 and ZFPAp3 showed even higher specificity than ZFPm2 and ZFPm3 (Fig. 7-11). M12 site is a sequence that contains overlapping m1 and m2 binding sites. Similarly, m34 contains overlapping m3 and m4 sequences.

To evaluate the specificity of new zinc finger protein, the affinity of each protein was determined for binding to the target sequence and several non-target sequences at appropriate protein concentration. For example, to protein ZFPm1 and ZFPm2, the non-target oligoes are: Oligo m34 (SEQ ID NO:20), Oligo Ap3 (SEQ ID NO:21), NRI-1 (SEQ ID NO:22), NRI-2 (SEQ ID NO:23), hHD-I (SEQ ID NO:24), hHD-II (SEQ ID NO:25), c5c1-g (SEQ ID NO:26), c5p3-g (SEQ ID NO:27), B3c2 (SEQ ID NO:28), and e2c-g (SEQ ID NO:29). Similarly, to protein ZFPm3 and ZFPm4, the non-target oligoes are Oligo m12 (SEQ ID NO:19), oligo Ap3 (SEQ ID NO:21), and the eight other oligoes. To protein ZFPAp3, the non-target oligoes are Oligo m12, oligo m34 (SEQ ID NO:20), and the eight other oligoes. The affinity reading for target oligo was further normalized to the no oligo control which has the least affinity. Table 3 showed the relative affinity of each zinc finger protein to the listed 11 oligo sequences. This relative affinity represents the fold of specificity of each protein to its target (specific or non-specific). The higher number of the fold of specificity means the more specific of this protein to that particular oligo. For ZFPm1, ZFPm2, ZFPm3, ZFPm4, and ZFPAp3, the fold of specificity to its own target oligo are 18, 23, 13, 16, and 27, respectively, the highest number among all 11 oligoes tested. The result indicates that the six-finger protein, as well as the three-finger proteins, bound their target oligo with significantly higher affinity than the non-target oligoes in every case.

Table 3. ZFP specificity

Protein (ug/ml)	Testing oligoes										
	Ap3	m12	m34	NRI-1	NRI-2	hHDI	c5c1-g	c5p3-g	B3c2	e2c-g	no oligo
AFPm1 (31.0)	4	18	5	5	4	5	4	7	3	4	2
ZEPm2 (15.5)	3	23	13	7	2	3	2	5	3	4	2
ZFPm3 (1.9)	7	3	13	4	6	1	3	3	3	3	1
ZFPm4 (15.5)	2	4	16	5	3	2	2	3	2	5	5
ZFPAp3 (31.0)	27	14	10	8	4	2	2	2	2	3	1

Refinements in binding affinity and specificity can be further obtained by random mutagenesis, as by PCR mutagenesis to introduce mutations in the ZFP to improve ZFP specificity or affinity.

5 C. DNA binding affinity of ZFPm1, ZFPm2, ZFPm3, ZFPm4, and ZFPAp3

The affinity of the purified six-finger proteins was measured using gel shift assay. The target oligos used in the ELISA assays were radioactively labeled using [γ 32P]-ddATP and terminal transferase. Ten serial dilutions of 1:3 with the purified protein (starting with ~1mg/ml) in binding buffer (1pM labeled oligo, 10% Glycerol, 0.8% BSA, 10 0.1ug/ μ l salmon sperm DNA in Zn Buffer A) were performed. The ZFP was allowed to bind at room temperature for 1hr. The samples were then run on 5-6% non-denaturing PAGE gel in TBE. The gel was dried and exposed with Phosphoimager. The affinity (Kd) of the ZFP was calculated from this gel. The Kd value is the concentration of the protein at which half of the labeled oligo is shifted to a higher molecular weight on the gel by binding to the ZFP. The affinities of these 6-finger proteins were determined to be: ZFPm1=2.0nM, ZFPm2=7.5 nM, ZFPm3= 0.18 nM, ZFPm4=0.25 nM and ZFPAp3=2.3 nM (Fig. 12-16). Most of these affinities compare favorably with the existed 6 finger proteins. The 2C7 derivative of the Sp1 zinc finger has a specificity of 0.46 nM and the e2c zinc finger constructed earlier (Beerli *et al.*, *Proc. Natl. Acad. Sci. USA* (1998) 95:14628-14633) has an affinity of 0.5 nM.

15 D. Evaluation of zinc finger protein ZFPm1, ZFPm2, ZFPm3, and ZFPm4 in reporter I system (*In vivo* characterization)

To evaluate the *in vivo* binding ability of the zinc finger proteins, ZFPm1, ZFPm2, ZFPm3, and ZFPm4 were cloned into plasmid reporter I system. Two steps are involved: First step is to modify the reporter construct by replacing 2C7 binding site with MIPS gene specific binding site m12 and m34. The second step is to modify the activators (activation constructs) by replacing the 2C7 fragment with each of these four new zinc finger proteins. To evaluate ZFPm1, the ZFPm1-VP64 activation construct is co-transformed with m12-luciferase reporter construct. The luciferase reading from this activation transformation is compared to the BG luciferase activity (no activator). The ZFPm2, ZFPm3, and ZFPm4 are evaluated using the similar steps.

The results show that all four new zinc finger proteins can mediate the activation of reporter gene. In this case, an endogenous target gene sequences were used as a heterologous target gene to activate the protein Luc fusion by piggy backing the VP64 regulator protein activator domain to a ZFP specific for the endogenous target gene sequence. The activation level is comparable to the 2C7 zinc finger protein (Fig. 17).

5 **Example 7**

10 **Evaluation of AP3-specific ZFP-activators with transient assays in**
***Arabidopsis* leaf protoplasts**

15 A. **Transformation of AP3-specific ZFP-activators into *Arabidopsis* leaf**
protoplasts

The ZFPAp3 domain was fused to the N terminal of the VP64 activation domain and then cloned into plant expression vector under the control of the UBQ3 promoter (Fig. 18).

20 For transient expression of AP3 activator in *Arabidopsis* protoplasts, cut 10 fully expanded *Arabidopsis* leaves into 1mm strips and add to 10ml enzyme solution (2.5 mg/ml Yakult Macerozyme R10, 15 mg/ml Yakult Cellulase R10, 0.4M Mannitol, 10 mM MES-KOH (pH 5.6), 19mM CaCl₂, 0.35 ul/ml beta-Mercaptoethanol, 0.1% BSA). Incubate 30 min in the dark at room temp under vacuum (run vacuum pump for 3-4 min then hold vacuum and turn off pump). Incubate 1.5 hours on orbital shaker at room temp 40 rpm, in the dark. Incubate 5 min on orbital shaker at room temp 80 rpm, in the dark. Filter protoplasts through 70 um nylon mesh. Dilute with 4 mL 200 mM CaCl₂. Spin at 60 g 4°C, 5 min. Remove supernatant. Re-suspend pellet in 3mL wash solution (333 mM Mannitol, 133mM CaCl₂). Spin at 40g for 5 minute 4°C. Remove supernatant.

25 Suspend pellet in 2 mL W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM Glucose, 1.5 mM MES pH 5.6 with KOH). Incubate on ice 30 min. Count cells. Spin in 40g, 4°C, 5 min. Remove supernatant. Re-suspend to 2 x 10⁶ cells per mL MMM solution (0.6 M Mannitol, 22.5 mM MgCl₂, 7.5 mM MES-KOH pH 5.6). Add 6ug of effector plasmid DNA to a 2ml centrifuge tube. Add 60 uL protoplast solution. Add 30 69uL of a 40% PEG, 400 mM Mannitol, 100 mM Ca(NO₃)₂ solution. Mix thoroughly but gently by rolling tube. Incubate 30 min. at room temp. Dilute with 200 uL W5

solution, mix gently, and incubate 10 min at room temp. Add 400 uL W5 mix gently, incubate 10 min. room temp. Add 800 uL W5, mix gently. Spin in microcentrifuge at 60 x g, room temp. for 5 min. Remove supernatant from all tubes. Re-suspend with 800 uL of 400mM mannitol/W5 4:1 solution. Spin in microcentrifuge at 60 x g, room temp. for 5 min. Remove supernatant. Re-suspend with 600 uL of culture medium (500 mM Mannitol, 10 mM KCl, 4 mM MES-KOH pH 5.6). Plate in 12 well Falcon culture plates. Incubate at room temp under fluorescent light for 24hrs. Harvest the protoplasts using a large bore pipette Spin in microcentrifuge at 400g, room temp, 5 min.

5 B. RT-PCR analysis of endogenous gene AP3 expression level

10 Extract RNA with Qiagen Plant RNeasy kit. RTPCR 200ng of RNA with Qiagen 1-step RTPCR kit in a 25ul reaction [5ul 5x Buffer, 1ul dNTP's, 0.125ul 100uM Ap3-F (SEQ ID NO:30) (5'-GGCGAGAGGGAAGATCCAG-3'), 0.125ul 1 100uM Ap3-4R (5'-CTCCTCTAATACGACTCACTATAGGGACACTCACCTAGCCTCTG-3') (SEQ ID NO:37), 1ul Enzyme mix]. Thermocycler setting: 50° C 30', 95° C 15', (94° C 30", 60° C 30", 72° C 1') x39 cycles, 72° C 10'.

15 C. PCR analysis of effector fusion protein expression level

20 Using the RNA prepared above, the amount of activator expression can also be determined. AP3-specific effector expression can be detected by RTPCR as described above using a generic zinc finger forward primer, NZlib5' (GGCCCAGGCAGGCCCTCGAGC) (SEQ ID NO:31) and an Ap3-specific reverse primer Ap3f4-R, (CTAACCAAGGAGCCACTGGTG) (SEQ ID NO:32). Similarly, the m4-specific effector expression can be detected using NZlib5' and a m4-specific reverse primer, m4f3-R (CCTCGCAAGATCACGACAATC) (SEQ ID NO:33).

25 D. Effects of expressing ZFPAp3-activator fusion protein on the transcriptional level of endogenous gene AP3

The AP3 gene is expressed exclusively in the developing flower, so no expression was expected in the leaf-derived protoplasts. Three constructs, AP3 activation (pND3014), m4 activation (pND3013) and GFP control (pND0001) were transformed into *Arabidopsis* leaf cells (protoplasts) respectively. The ZFPm4-specific constructs was used as a control in this experiment to show that the gene specific transcription factors generated with this technology affect only their target gene since the ZFPm4-specific

activator (ZFPm4-VP64) should have no effect on the endogenous AP3 gene. The result show that only in the cells that are transformed with AP3 activation constructs there are AP3 transcripts detected (Fig. 19). Thus the ZFP domain is able to direct the activation domain (VP64) to the specific endogenous target gene.

5

Example 8

Stable repression and stable activation of AP3 gene expression in transgenic *Arabidopsis*

A. Transformation of AP3-specific ZFP-repression construct into *Arabidopsis* plant

An *Agrobacterium* transformation vector that contains expression cassette of UBQ3::SID-ZFPAp3//nos with hygromycin as selection marker was created and named as pND0051. Similarly, expression cassette of UBQ3::ZFPAp3-VP64//nos (ZFPAp3 activation construct) was cloned into this *Agrobacterium* transformation vector as well and named as pND0052 (Fig.18). These plasmids were then transformed into *Arabidopsis* plants using *Agrobacterium*-mediated transformation methods. About 20 transgenic plants were generated.

B. PCR analysis of effector fusion protein expression level

The expression of ZFPAp3 gene in each transgenic event was identified by RT-PCR (Example 7, section C).

C. RT-PCR analysis of endogenous gene AP3 expression level

The transcriptional level of endogenous AP3 gene in each transgenic event was detected by RT-PCR (Example 7, section B).

D. Quantitative analysis of AP3 gene expression levels by Quantitative PCR

The TaqMan assay was used for quantifying AP3 gene expression in transformed *Arabidopsis* plant. The quantitative PCR probe for AP3 (SEQ ID NO:34) and two primers (SEQ ID NO:35 and SEQ ID NO:36) were designed using Primer Express (Perkin Elmer). RNA samples were prepared from average of two flower heads using RNAwiz method (Example 7, Section B). TaqMan PCR reaction was done using 250ng total RNA and TaqMan One-step RT-PCR Master mix reagent. Thermal cycling conditions are: 48°C, 30min.; 95°C, 10min.; then followed by 40 cycles of 95°C, 15''; 60°C, 1min.. Data were analyzed using relative quantification of the comparative Ct

method. For comparative Ct method, thioredoxin3 gene was used as a relative internal standard at this time.

5 E. Effects of expressing ZFPAp3-repressor fusion protein on the transcriptional level of endogenous gene AP3

Both RT-PCR and quantitative PCR with AP3 specific primer was performed on these transgenic events (above example 7A). The endogenous gene AP3 expression level has been changed in several events, especially for three events: ND0052-2e, ND0052-257, and ND0051-1a.

10 Plant ND0052-2e contains very high expression level of ZFPAp3-SID transgene (Fig. 20A). The transcriptional level of endogenous AP3 gene has been significantly down regulated (Fig. 20B). Quantitative analysis shows a nearly 50 fold of repression was achieved in this plant (Fig. 21).

15 The transcriptional level of endogenous AP3 gene in Plant ND0052-257 is less repressed compared to plant ND0052-2e, about 5 fold (Fig. 21).

20 Only one event containing activation construct was confirmed and designated as ND0051-1a. Curiously, there was a two-fold activation of the endogenous AP3 gene in this plant (Fig. 21). The phenotype of this plant looks normal. We have not been able to detect the protein expression level in this plant. Considering the significantly less number of activation events that were finally identified (ten to twenty times less than the number of repression events, Table I), it is reasonable to assume that over-activation and over-expression of AP3 gene is toxic to the plant. This toxic effect could cause loss of transgenic events during selection and regeneration. Consistent with this notion is that our transient regulation data indicates that activation is much more efficient than repression. Expression of ZFPAp3-VP64 construct with a weaker promoter or with an inducible promoter would or might increase transgenic events.

25 F. Phenotype changes caused by expressing ZFPAp3-repressor fusion protein in transgenic *Arabidopsis*

30 Among the repression events, there are three independent events that showed significant phenotype changes: flower structures and fertility. They are named as ND0052-2e, ND0052-2d, and ND0052-257. Molecular analysis has been performed on the ND0052-2e and ND0052-257 (Example 8, section E).

The first event, ND0051-2e, did not have a typical flower formation compared to the wildtype flower: the petals are under developed (short and narrow) and partially converted to sepal-like structure; the stamens are much smaller in size compared to the wildtype plant. However, there may be some pullens on the anther. The carpel appears normal except that the size is significantly bigger and developed much faster than the stamens. Generally, the phenotype of this flower is similar to the flowers from previously characterized mutant *ap3* (Jack *et al.*, *Cell* (1992) 68:683-687, Jack *et al.*, *Cell* (1994) 76:703-716) and *sap* (Byzova *et al.*, *Gene and Development* (1999) 13:1002-1014) but is definitely not identical. This plant is sterile. Comparing with the morphology of this flower with the wild type flower, it appears that the un-proportional development of stamen and stigma is the cause of sterility. More detailed analysis can be carried out in this area.

The second event is named as ND0052-257. This is a sterile plant too. The flower is very similar to flower from ND0052-2e. However, there seems missing one petal in the two flowers that we have dissected.

The third event is named as ND0051-2d. Same as the previous two, it is a sterile plant. There is no silique formation in this plant. The plant died during flowering stage.

Wild type flowers have four organ types (sepal, petal, stamen, carpel) arranged in concentric whorls. The AP3 gene is involved in the determination of organ identity. Misexpression of the AP3 gene results in homeotic mutations where whorl-specific organ identity will be altered. While we expected the phenotypes of the activated and repressed AP3 plants that generated through zinc finger protein technology should mimic the phenotype of previously described AP3 overexpressors, *ap3* mutants (Jack *et al.*, *Cell* (1992) 68:683-687, Jack *et al.*, *Cell* (1994) 76:703-716), and *sap* (Byzova *et al.*, *Gene and Development* (1999) 13:1002-1014), we also think the phenotype can not be identical since the mechanism of repression AP3 gene is different.

The promoter driving the expression of zinc finger protein is ubiquitin3 (UBQ3) promoter. UBQ3 is a constitutive promoter and the expression pattern and strength is very different from the native AP3 promoter.

We are analyzing more transgenic plants (both repression events and activation events) now. We will choose several plants (4 to 5) with different expression level of zinc

finger protein through Western analysis. The level of AP3 expression can then be correlated with the floral phenotype and effector expression levels.

AP3 gene is one of the many genes that are involved in floral organ determination (Weigel, *Annu. Rev. Genetics* (1995) 29:19-39; and Pineiro and Coupland, *Plant Physiol* (1998) 117:108). It is very likely that there are other genes, especially the down stream genes, that play a role in this very complex process as well. We plan to conduct gene chip assay (Affymetrix) on the final selected AP3 events. This assay should provide us with more insight into the floral development and regulation process.

More sterile plants have been identified other than the three events mentioned above. It is estimated that the chance of getting sterile phenotype with our repression construct is about 5 to 10%.

Example 9

Stable activation of AP3 gene expression in transgenic *Arabidopsis* with floral specific promoter AP3

Arabidopsis AP3 promoter region (1.9kb) was isolated by PCR according to Irish and Yamamoto (*Plant Cell* 7 (10), 1635-1644 (1995)). This fragment was used to replace the UBQ3 fragment in the pND0052 construct. The final construct was named as pND3045 (AraAP3 promoter::ZFPAp3-VP64//nos). pND3045 was transformed into *Arabidopsis* as described in previous section. About 400 plants were generated through selection media. There are several plants that have shown sterile phenotype already. Molecular analysis on these events can be performed as previously described.

High numbers of transgenic plants have been easily selected with this tissue specific activation construct. With our previous activation construct pND0051 (with constitute promoter UBQ3), we only obtained three transgenic plants and none of them shows high level of ZFP expression and none of them shows phenotypic changes. These results support our previous hypothesis that over-activation and over-expression of AP3 gene is toxic to the plant.

Example 10

Evaluation of regulation of endogenous gene expression using the zinc finger proteins having less than six fingers

The Ap3-VP64 6-finger construct appears to work effectively and specifically to activate and repress AP3 expression in Arabidopsis protoplasts or even the whole plant. This is a good system to test the activity of zinc finger protein with less than 6 fingers, such as 3-finger effector constructs. The 6-finger ZFPAp3 protein was constructed from two 3-finger proteins (See Example 3). ZPFAp3a is a 3-finger protein with fingers 4-6 of ZFPAp3. Similarly, ZPFAp3b has fingers 1 to 3 of ZFPAp3.

To test these 3-finger constructs, the Ap3-VP64 expression plasmid is digested with SfiI to remove the 6-finger Ap3 coding region. The 300bp SfiI fragment of the AP3 3-finger proteins is ligated in this digested vector to generate Ap3a-VP64 and Ap3b-VP64 constructs. The activation of AP3 expression in protoplasts is tested with these constructs using the method described in Example 7 and 8. Generally, 3-finger protein is less specific than the 6-finger protein. But, this may not be the case for all the targets (genes). If some of these 3-finger effectors are able to specifically and effectively activate AP3 expression, this will indicate that 6 finger proteins are not required for all targets. This will also demonstrate the versatility of this technology with respect to the number of zinc fingers required.

Example 11

Evaluation of ZFP-activator/repressor fusion protein function in maize

MIPS is an endogenous maize enzyme. Its gene expression level can be monitored at three levels after transforming the maize cells with ZFPmips-activator or ZFPmips-repressor. The first level is on the transcriptional level. Quantitative PCR can be used to analyze the abundant of MIPS transcripts. The second level is on the protein expression level. We have generated MIPS specific antibody and can analyze the amount of MIPS protein expressed on Western blot. The third level is on the function level of MIPS enzyme. The activity of MIPS enzyme can be monitored by the concentration of its

product, phytic acid through HPLC (Talamond *et al.*, *J. of Chromatography* (1998) 805:143-147) and colorimetric method (Sigma, #670-A).

A. Construction of maize MIPS-specific ZFP-effectors

Four activation constructs were generated for ZFPm1, ZFPm2, ZFPm3, and ZFPm4 (ZFPmips) by fusing the ZFPmips domain to the N terminal of the VP64 activation domain. Two repression constructs were generated for ZFPm1 and ZFPm4 by fusing the ZFPmips domain to the C terminal of the SID repression domain. Then the ZFPmips-effector cassette was cloned into monocot expression vector under the control of the maize ubiquitin (ZmUbi) promoter (same as in pND3008). A "nos" fragment was placed at the 3'end of this cassette as transcription terminator (same as in pND3008). These six constructs are named as pND3015, pND3023, pND3024, pND3016, pND3019, and pND3017 (Fig.22).

Our transient regulation result in maize cells indicates that activation is more efficient than repression. So we have transformed all eight constructs into maize cells. A successful activation of MIPS gene in maize is an indication of success of this zinc finger protein technology in regulating another endogenous gene in another plant species. Maize is monocot and considered as crop plant with MIPS as an agronomic trait.

B. Function of ZFPmips-effectors in maize

A maize type II cell line HE89 was used to evaluate the ZFPmips-effector fusion constructs function (Fig.22). Protoplasts from HE89 suspension cells were isolated and transformed using standard procedures (Chourey and Zurawski, 1981). The activation constructs pND3015, pND3023, pND3024, and pND3016 were transformed into freshly prepared protoplasts. The transcription level of MIPS gene was detected through quantitative PCR. With pND3016 (ZFPm4-VP64 activation constructs), at least 2-fold of activation is detected (Fig. 23). We also measured phytic acid concentration in maize tissues that were transformed by the activation construct of pND3016. We found that the phytic acid concentration is increased from 304 pg/ml to 569 pg/ml (detection was carried out with HPLC).

We have demonstrated herein that zinc finger protein approach can be used in both up-regulation (as example of AP3 in Arabidopsis, MIPS in maize) and down-regulation (as example of AP3 in Arabidopsis) of endogenous gene expression in plant.

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The above examples are included for illustrative purposes only and is not intended to limit the scope of the invention. Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

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